

AN ABSTRACT OF THE THESIS OF

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Title: An Improved Method for the Isolation of Δ^2 -IPP:tRNA Isopentenyltransferase Activity from *Saccharomyces cerevisiae*.

Abstract approved

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Dr. Donald J. Armstrong

An improved method for the isolation of Δ^2 -IPP:tRNA isopentenyltransferase activity from *Saccharomyces cerevisiae* has been developed. The procedure involves precipitation of the enzyme activity from clarified crude yeast homogenates by addition of 1/15 volume of 10% (v/v) Polymyxin P and extraction of the enzyme from the precipitate with a buffer containing 0.3 M KCl. This Δ^2 -IPP:tRNA isopentenyltransferase preparation also contains most of the IPP isomerase present in the homogenate and at this stage of purification addition of an external isomerase source is not required for the isopentenylation of tRNA.

The Polymyxin P precipitation method has also been applied to the preparation of IPP isomerase from pig liver and wheat germ. In these systems, IPP isomerase activity remains in the supernatant fraction following the addition of Polymyxin P. The isomerase activity may be further purified by ammonium sulfate fractionation of the Polymyxin P supernatant to obtain a preparation enriched in IPP isomerase and with very low phosphatase content.

This procedure is more rapid and convenient than the most commonly used method for the preparation of IPP isomerase-active samples.

An Improved Method for the Isolation of
 Δ^2 -IPP:tRNA Isopentenyltransferase Activity
from Saccharomyces cerevisiae

by

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LIST OF ABBREVIATIONS

A: adenosine

bzl⁶Ade: N⁶-benzyladenine

C: cytidine

DE 23: diethylaminoethyl cellulose

i⁶Ade: N⁶-(Δ^2 -isopentenyl)adenine

i⁶Ado: N⁶-(Δ^2 -isopentenyl)adenosine

io⁶Ade: zeatin

io⁶Ado: ribosylzeatin

IPP: isopentenyl pyrophosphate

mRNA: messenger ribonucleic acid

ms²i⁶Ado: 2-methylthio-N⁶-(Δ^2 -isopentenyl)adenosine

ms²i⁶Ado: 2-methylthioribosylzeatin

MVA: mevalonic acid

PEG: polyethylene glycol

TCA: trichloroacetic acid

tRNA: transfer ribonucleic acid

TTAB: tetradecyltrimethylammonium bromide

U: uridine

AN IMPROVED METHOD FOR THE ISOLATION OF
 Δ^2 -IPP:tRNA ISOPENTENYLTRANSFERASE ACTIVITY
FROM SACCHAROMYCES CEREVISIAE

I. INTRODUCTION

The occurrence of cytokinin-active ribonucleosides in transfer ribonucleic acid (tRNA) molecules has been known since 1966 (131). All organisms examined to date, except one species of *Mycoplasma* (45), have cytokinin-active ribosides in at least one tRNA species (109). The cytokinin-active ribonucleosides that occur in tRNA include N⁶-(Δ^2 -isopentenyl)adenosine (i⁶Ado) and structurally related compounds. The relationship between the cytokinin-active constituents of tRNA molecules and the hormonal function of free cytokinins in plant tissue remains unresolved.

The cytokinin-active ribonucleosides in tRNA arise by direct isopentenylation of the appropriate adenosine residues in pre-formed tRNA molecules (18,26,27,65,99). The enzyme involved in this reaction transfers the isopentenyl group from Δ^2 -isopentenylpyrophosphate (Δ^2 -IPP) to unmodified adenosine residues adjacent to the 3'-end of the anticodon in certain tRNA species that correspond to codons beginning with uridine (U) (55,56,105). Δ^2 -IPP:tRNA isopentenyltransferase has been partially purified from several organisms (9,27,55,56,65,105).

Several methods have been employed to obtain Δ^2 -IPP:tRNA isopentenyltransferase activity in cell-free preparations. The simplest involve clarification of crude homogenates by centrifugation (27). More complex methods involving various combinations of ammonium sulfate precipitation, gel filtration, ion-exchange chromatography and polyethylene glycol-dextran extraction have been utilized to obtain purified preparations of the enzyme (9,56,57,65,105). Assay for Δ^2 -IPP:tRNA isopentenyltransferase activity in crude homogenates is difficult due to the labile nature of the substrates. The present

study was undertaken to develop a simple and rapid procedure for the partial purification and routine assay of the enzyme. Polyethylenimine (Polymine P), a compound that has proven useful in the purification of RNA polymerase II (60), has been tested for its utility in the assay of transferase activity. The original objective of this investigation was to develop a procedure that could be employed with plant tissue, but initial difficulties in obtaining transferase activity from plant sources led to the selection of an alternative system for methodological investigations. The organism selected as a model system for these studies was Saccharomyces cerevisiae. Yeast tRNA has a relatively high level of isopentenyl groups, and Δ^2 -IPP:tRNA isopentenyltransferase can be readily obtained from yeast cells.

Cell-free assay of the partially purified Δ^2 -IPP:tRNA isopentenyltransferase requires the presence of IPP isomerase because only the Δ^3 -IPP isomer is commercially available. Most Δ^2 -IPP:tRNA isopentenyltransferase assays have been coupled with IPP isomerase purified from pig liver according to the method of Holloway and Popjak (52,53). Procedures and sources for the preparation of IPP isomerase have also been examined in the present study.

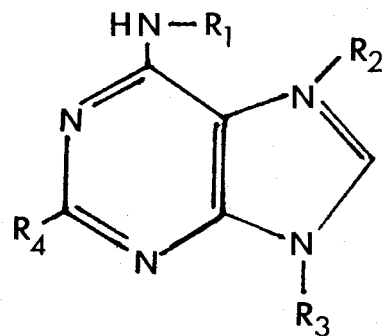
II. REVIEW OF LITERATURE

Naturally Occurring Cytokinins

The term "cytokinin" is a generic name for substances which promote cell division and exert certain other plant growth regulatory functions exhibited by kinetin (109).

All cytokinins that have been unequivocally established as naturally occurring compounds are N⁶-substituted adenine derivatives. Certain compounds that are not adenine derivatives, for example diphenylurea (108) do exhibit kinetin-like activity, but it is not certain whether these compounds occur naturally or not, or even if their mode of action is similar to that of the cytokinin-active N⁶-substituted adenines. With one exception, all of the naturally occurring cytokinins identified to date bear N⁶-isoprenoid side chains. The one exception is N⁶-(o-hydroxybenzyl)adenosine, isolated from poplar (59). The N⁶-isoprenoid side-chains found in naturally occurring cytokinins include the 4-hydroxy-3-methyl-trans-2-butenyl, 4-hydroxy-3-methyl-cis-2-butenyl, 3-methyl-2-butenyl, 4-hydroxy-3-methyl-butyl, 3,4-dihydroxy-3-methylbutyl, and 2,3,4-trihydroxy-3-methylbutyl groups (22,57,73,82,92,101,127). In addition to the N⁶-side chain, naturally occurring cytokinins may also have substituent groups at the 2, 7 and 9 positions of the adenine ring. At the 2 position, the known substituents are hydroxyl and methylthio groups. Glucose molecules may be attached at position 7, and glucose or ribose moieties may be present at position 9 (67,92-94,97,111,121). A purine-amino acid conjugate called lupinic acid, identified as L-β-[6-(4-hydroxy-3-methyl-trans-butenylamino)-purin-9-yl]alanine, has been isolated by Parker and associates (95), and cytokinin riboside-5'-mono-, di- and triphosphates have been demonstrated to be synthesized in higher plants (67,68). A list of some of the naturally occurring cytokinin-active compounds identified to date is given in Table 1. Some are probably degradation products, precursors, or even storage forms of the physiologically-active cytokinins. The relationship among these various naturally occurring cytokinin-active com-

Table 1. Structural formulae and names of the common naturally-occurring cytokinins.



<u>Structure</u>				<u>Chemical Name</u>	<u>Common Name</u>
R ₁	R ₂	R ₃	R ₄		
$-\text{CH}_2\text{CH}=\text{C}\begin{smallmatrix} \text{CH}_3 \\ \text{CH}_3 \end{smallmatrix}$	-H	-H	-H	N ⁶ -(Δ ² -isopentenyl)adenine	Isopentenyladenine
$-\text{CH}_2\text{CH}=\text{C}\begin{smallmatrix} \text{CH}_3 \\ \text{CH}_3 \end{smallmatrix}$	-H	-ribose	-H	N ⁶ -(3-methyl-2-butenylamino)- 9-ribofuranosylpurine N ⁶ -(Δ ² -isopentenyl)adenosine	Isopentenyladenosine
$-\text{CH}_2\text{CH}=\text{C}\begin{smallmatrix} \text{CH}_3 \\ \text{CH}_3 \end{smallmatrix}$	-H	-ribose	-CH ₂ SH	N ⁶ -(3-methyl-2-butenylamino)- 2-methylthio-9-ribofuranosyl purine	Methylthio-isopen- tenyladenosine
$-\text{CH}_2\text{CH}=\text{C}\begin{smallmatrix} \text{CH}_2\text{OH} \\ \text{CH}_3 \end{smallmatrix}$	-H	-H	-H	N ⁶ -(4-hydroxy-3-methyl-2- butenyl)-adenine	<u>trans</u> -Zeatin
$-\text{CH}_2\text{CH}=\text{C}\begin{smallmatrix} \text{CH}_2\text{OH} \\ \text{CH}_3 \end{smallmatrix}$	-H	-ribose	-H	N ⁶ -(4-hydroxy-3-methyl-2- butenylamino)-9-ribofura- nosyl purine	<u>trans</u> -Ribosylzeatin

<u>Structure</u>				<u>Chemical Name</u>	<u>Common Name</u>
R ₁	R ₂	R ₃	R ₄		
$-\text{CH}_2\text{CH}=\text{C}\begin{matrix} \text{CH}_2\text{OH} \\ \text{CH}_3 \end{matrix}$	-H	-ribose	$-\text{CH}_2\text{SH}$	N ⁶ -(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-ribofuranosyl purine	Methylthio- <u>trans</u> -ribosylzeatin
$-\text{CH}_2\text{CH}=\text{C}\begin{matrix} \text{CH}_2\text{OH} \\ \text{CH}_3 \end{matrix}$	-glucose	-H	-H	N ⁶ -(4-hydroxy-3-methyl-2-butenylamino)-7-glucopyranosyl purine	7-Glucosylzeatin (Raphanatin)
$-\text{CH}_2\text{CH}=\text{C}\begin{matrix} \text{CH}_2\text{OH} \\ \text{CH}_3 \end{matrix}$	-H	-glucose	-H	N ⁶ -(4-hydroxy-3-methyl-2-butenylamino)-9-glucopyranosyl purine	9-Glucosylzeatin
$-\text{CH}_2\text{CH}=\text{C}\begin{matrix} \text{CH}_2\text{O-glucose} \\ \text{CH}_3 \end{matrix}$	-H	-H	-H	N ⁶ -(4-glucopyranosyloxy-3-methylbutylamino)purine	0-Glucosylzeatin
$-\text{CH}_2\text{CH}=\text{C}\begin{matrix} \text{CH}_2\text{OH} \\ \text{CH}_3 \end{matrix}$	-OH	-H	-H	N ⁶ -(4-hydroxy-3-methyl-2-butenylamino)-2-hydroxy-purine	2-Hydroxyzeatin
$-\text{CH}_2\text{CH}_2\text{CH}\begin{matrix} \text{CH}_2\text{OH} \\ \text{CH}_3 \end{matrix}$	-H	-H	-H	N ⁶ -(4-hydroxy-3-methyl-butyl-amino)purine	Dihydrozeatin
$-\text{CH}_2-\text{C}_6\text{H}_4-\text{OH}$	-H	-ribose	-H	N ⁶ -(0-hydroxybenzylamino)-9-ribofuranosyl purine	Hydroxybenzyl-adenosine

pounds and the physiological importance of each are still not fully understood. In terms of cytokinin activity, the free base forms are usually the most active. The cytokinin ribosides generally have a lower level of cytokinin activity than their corresponding free bases (47). This also appears to be true for the glucosides. In the soybean bioassay technique, for instance, Laloue and coworkers (67) found the cytokinin activity of benzyladenine-7-glucoside to be much lower than that of benzyladenine. In fact, evidence seem to suggest that cytokinin 7-glucosides are storage forms of cytokinins (31,69)

Metabolism of Cytokinins

The biosynthetic pathway of free cytokinins is still not completely certain. The presence of cytokinin-active ribonucleosides in tRNA (40,62,109) led Chen and Hall (18) to suggest that free cytokinins might be derived from tRNA molecules. If indeed tRNA is the only source of free cytokinins then it becomes necessary to show that tRNA turnover is rapid enough to account for the free cytokinin level in plant cells. Half-life studies on tRNA from Agrobacterium tumefaciens (37), primary roots of Zea mays (64), and Lactobacillus acidophilus (49,72) are compatible with this possibility, but estimation of the tRNA half-life of tobacco cells was found, in contrast, to be too long to account for all of the free cytokinin (118).

Evidence for a tRNA-independent pathway of cytokinin biosynthesis has been obtained by Chen et al. (17). These investigators supplied cytokinin-autonomous tobacco tissue with a ^{14}C - and ^3H -labeled adenosine analog, 2-O-[1-(R)-(9-adenyl)-2-(hydroxy)ethyl]glycerol. This analog was not incorporated into tRNA, but the corresponding isopentenylated analog was still synthesized by the tissue. Further evidence for a tRNA-free pathway comes from a recent report by Burrows (12) that dihydrozeatin and N^6 -(2-hydroxybenzyl)adenosine, free cytokinins found in Lupinus luteus and Populus x robusta respectively, could not be found in the tRNA of these plants. Holtz (54) raised the possibility that free cytokinins may come from the isopentenylation of oligonucleotides. The supporting evidence, however, is not very convincing. The most convincing evidence for a tRNA-independent biosynthesis of

cytokinins was obtained by Taya *et al.* (117). They were able to demonstrate N⁶-(Δ²-isopentenyl)adenine (i⁶Ade) synthesis in a cell-free system from *Dictyostelium discoideum*. They showed that 5'-adenosine monophosphate was the actual acceptor molecule of the isopentenyl group.

Most studies on the interconversion and degradation of cytokinins have been done by supplying cells with exogenous cytokinins. Early work of this nature was conducted by Sondheimer and Tzou (111) who found that exogenous zeatin (io⁶Ade) was metabolized by bean axes to zeatin riboside, zeatin-5'-monophosphate and dihydrozeatin derivatives. Both Hall (40) and Paces (90) consider i⁶Ado to play a central role in the metabolism of free cytokinins. In animal tissues, i⁶Ado can be readily converted to inosine (41). A cytokinin oxidase from plant tissues cleaves the isopentenyl group from i⁶Ado to give adenosine (89,91,130). In detached barley leaves (88) and in germinating rape seeds (90) i⁶Ado yields trans-ribosylzeatin (trans-io⁶Ado) (80,82). In turn, io⁶Ado can be converted to zeatin or to adenosine (130). Work done by Laloue and his associates using exogenous benzyladenine (67-70) showed that the first metabolites formed by wheat tissue were the ribosides and the mono-, di- and triphosphates ribosides. Subsequently, there was a rapid decline in the levels of these nucleosides and a gradual increase in the 7-glucosides. The nucleotides were found to be formed more readily from the ribosides than from the free base, while the reverse was true in the case of 7-glucoside formation. Letham and coworkers have also contributed substantially to our knowledge on cytokinin metabolism (22,23,35,73,92-97). In de-rooted radish seedlings zeatin was found to be metabolized to numerous products including degradation products such as adenine, adenosine and adenosine monophosphate, the riboside and riboside monophosphate derivatives of zeatin, and later, 7-glucosylzeatin, 0-glucosylzeatin, 0-glucosylpyranosyldihydrozeatin and 0-glucosyl-9-ribofuranosyldihydrozeatin.

The 7-glucosides have been suggested to be the active form of cytokinins (28), a storage form of cytokinins (31,35,92), or detoxification products. More recent data (31,35,93) strongly point to a sto-

rage role for the 7-glucosides. The O-glucosides have been shown to be the predominant cytokinins in leaves actively accumulating cytokinins (122,127) and in plant tumor cells grown under conditions which increase cytokinin production (101). The fact that the riboside of zeatin is the only zeatin metabolite of any significance to be detected in xylem sap points to a translocation role for the ribosides (11,35). Similarly, $i^6\text{Ado}$ has also been found in xylem sap (unpublished of Dyson *et al.* cited in 40).

Cytokinin Constituents on tRNA Molecules

(a) General

Transfer RNA molecules are characterized by a high content of modified bases. In fact, more than 50 different types of modified nucleosides are known to occur in tRNA molecules (79). These modifications range in complexity from methylation of the 4 common ribonucleosides to moieties involving ring structure modification. These modified bases are usually found in the unpaired regions of the tRNA molecule. The significance of these modified bases is not completely understood. It is conceivable that they play important roles in determining the *in vivo* configuration of the tRNA molecule, in recognition and binding of the correct aminoacyl-tRNA synthetase, and in the pairing of the anticodon to the complementary codon of the messenger RNA (mRNA).

(b) Types of cytokinin modifications found in tRNA preparations

Soon after the appearance of the first report of a complete tRNA sequence by Holley *et al.* (51), Zachau and associates (131) and Biemann *et al.* (10) published the tRNA sequence of two yeast tRNA^{Ser} species. Of interest here was the discovery of the highly active cytokinin, $i^6\text{Ado}$, adjacent to the '3' position of the anticodon in these tRNA^{Ser} molecules. Soon thereafter, tRNA hydrolysates from yeast and calf liver were found to contain $i^6\text{Ado}$ (43,104), and *Escherichia coli* tRNA hydrolysates were shown to be cytokinin-active (110). *cis*-Ribosylzeatin and $i^6\text{Ado}$ were subsequently isolated from plant tRNA (42). The cytokinin, 2-methylthio- N^6 -(Δ^2 -isopentenyl)adenosine ($\text{ms}^2.i^6\text{Ado}$) was first isolated by Burrows and coworkers in 1968 from *E. coli* tRNA (14), and studies

on wheat germ tRNA by Skoog and associates (48) yielded the first evidence of methylthioribosylzeatin (ms^2io^6Ado). In fact, wheat germ tRNA was later found to contain three other cytokinin-active ribonucleosides: i^6Ado , io^6Ado and ms^2i^6Ado (13).

Until 1972, it was believed that in tRNA both io^6Ado and its methylthio derivatives possessed the cis-configuration exclusively, while their corresponding free base forms had the trans-configuration. Vreman and coworkers (125), however, obtained evidence for the existence of small amounts of the trans-zeatin isomer in tRNA. Indeed, trans- io^6Ado is the major cytokinin-active ribonucleoside present in the tRNA from at least some strains of Agrobacterium tumefaciens (16). To date, there is one report of the occurrence of the cis-isomer in the free form in plant material (76), and it has been identified in the culture filtrate of Corynebacterium fascians, a plant pathogen (106).

In all organisms examined to date, except one species of Mycoplasma (45), at least one species of tRNA contains cytokinin-active ribonucleosides. Apparently, tRNA molecules from animal sources possess i^6Ado as the only cytokinin-active ribonucleoside (e.g. 129). The major tRNA cytokinins in most microorganisms are i^6Ado and ms^2i^6Ado (4, 36, 109). However, microorganisms associated with plants (either symbiotically or as pathogens) may have in addition ms^2io^6Ado and io^6Ado in their tRNA molecules (16, 19, 63, 76, 102, 119). In higher plants the most common cytokinin in tRNA is io^6Ado and the least common is ms^2i^6Ado (6, 12, 14, 42, 115, 124). The other two tRNA cytokinins, i^6Ado and ms^2i^6Ado are present in intermediate amounts.

Studies on green versus non-green plant tissue (42, 124) and light versus dark-grown and chloroplastless Euglena mutants (115, 117) seem to indicate that the methylthiolated cytokinins may be associated with organelle tRNA. Conversely, Vreman et al. (126) could readily detect io^6Ado in spinach leaves but not in isolated chloroplasts.

(c) Cytokinin distribution among tRNA species

The levels of cytokinin-active ribonucleosides found in tRNA preparations (10, 43, 104, 131) are not sufficient for this type of base modification to occur in all tRNA molecules. The distribution of cyto-

kinins in the tRNA species of yeast (5,10,48,75,131), E. coli (3,86), Staphylococcus epidermidis (4), Lactobacillus acidophilus (100), Drosophila (129), and wheat germ (113) have been investigated in some detail. In all of these organisms, cytokinin-active ribonucleosides were found only in tRNA species that correspond to codons that begin with uridine. Sequence analysis of cytokinin-containing tRNA species, for example of yeast and rat liver tRNA^{Ser}_{UCY} (112,131), yeast tRNA^{Tyr} (75,87) and E. coli tRNA^{Phe} (8,121), has demonstrated that the cytokinin moieties occur adjacent to the 3' end of the anticodon.

Transfer RNA molecules that have anticodons complementary to codons beginning with U are tRNA^{Cys}, tRNA^{Leu}, tRNA^{Phe}, tRNA^{Ser}, tRNA^{Trp} and tRNA^{Tyr}. Serine and leucine have codons beginning with adenosine (A) and cytidine (C) respectively as well as with U. Transfer RNA^{Ser} species which contain cytokinins (4,5,112,125,129,131) all correspond to codons beginning with U, while those species which correspond to codons beginning with A do not contain cytokinins (3,4,129). Similarly, tRNA^{Leu}_{UUG} molecules contain cytokinins, but neither tRNA^{Leu}_{CUY} nor tRNA^{Leu}_{CUX} molecules contain cytokinin moieties (3,85).

It does not follow, however, that all tRNA species corresponding to codons beginning with U contain cytokinins. In fact, analogous tRNA species may contain cytokinins in one organism and not in another. For example, yeast tRNA^{Trp} (61), tRNA^{Phe} (110) and tRNA^{Tyr} (5) species, and wheat germ tRNA^{Trp}, tRNA^{Cys}, tRNA^{Tyr} and tRNA^{Phe} species (113) all lack cytokinin-active ribonucleosides. In the case of many eukaryotic tRNA^{Phe} species, the base adjacent to the 3'-position of the anticodon is actually base Y, a modified guanosine derivative (21,103,84).

(d) Biosynthesis of cytokinin moieties in tRNA

The first indication that the isopentenyl side-chain of i⁶Ado found in tRNA is derived from the isoprenoid biosynthetic pathway came from work done by Fittler et al. (26) and Peterkofsky (99). These early studies involved in vivo incorporation of labeled mevalonic acid (MVA) into Lactobacillus. Crude cell-free extracts from yeast and rat liver (27) and from tobacco pith tissue (18) were subsequently shown to have the ability to catalyze the incorporation of label from MVA

into ⁶Ado residues in tRNA molecules. The enzyme involved, Δ^2 -IPP:tRNA isopentenyltransferase, was partially purified from yeast (65). This enzyme was shown to specifically require the Δ^2 -isomer of isopentenyl pyrophosphate as a substrate for the isopentenylation of tRNA molecules (9,39,55,56,65,105). Unfortunately, the Δ^2 -isomer is not available commercially. In practice, the commercially available Δ^3 -isomer is converted to the Δ^2 -isomer by IPP isomerase (1,7,20,52,53,107). Thus the isomerase enzyme must be present in the reaction mixture in order to conduct in vitro studies on partially purified Δ^2 -IPP:tRNA isopentenyltransferase.

Most native tRNAs are not good substrates for the isopentenylation reaction, because practically all the tRNA molecules that can be isopentenylated already have this side chain. To circumvent this problem, Hall and coworkers (65) removed the isopentenyl side-chain from the tRNA molecules by a mild treatment with potassium permanganate. Mild permanganate treatment does, however, also cause unwanted oxidation of a small but substantial amount of pyrimidine residues (46) and somewhat decreases the amino acid acceptor activity of the tRNA molecules (58, 65). Furthermore, in the case of E. coli tRNA, permanganate-treated tRNA is a poorer substrate for the transferase enzyme than untreated tRNA (58,123). However, in most cases, mild permanganate treatment of tRNA results in an acceptable substrate for the isopentenylation reaction (27,56). Alternatively, naturally-occurring undermodified tRNA has been isolated from Mycoplasma and E. coli and used as substrate for isopentenylation in cell-free systems (9,105).

Δ^2 -IPP:tRNA isopentenyltransferase activity has now been detected in yeast (27,58,65,123), E. coli (9,105), Lactobacillus (26,27,55,99), tobacco pith tissue (18), rat liver (65), Zea mays (56) and Agrobacterium (Armstrong and Taller, unpublished data). The enzyme purified from E. coli was shown to have an approximate molecular weight of 55,000 daltons, to require reduced sulfhydryl groups, a divalent cation, tRNA, and Δ^2 -IPP. A pH of 7.5 was required for full activity (9,105). The requirements for optimal activity of the Lactobacillus (55), corn (56) and yeast (58) enzyme are similar to those of the E. coli enzyme. The molecular weight of the enzyme from corn was also in agreement with

that reported for the E. coli enzyme (56). In the case of corn, the enzyme obtained from different tissues preferentially isopentenylated tRNA from the same tissue (56). Kline et al. (65) have demonstrated isopentenylation in heterologous systems using rat liver and yeast Δ^2 -IPP:tRNA isopentenyltransferases and E. coli tRNA as a substrate.

Holtz and Klambt (56) have noted some rather unusual properties of the corn Δ^2 -IPP:tRNA isopentenyltransferase. When crude homogenates were centrifuged at 3000 x g the enzyme activity was observed in the supernatant, but when centrifugation was at 10,000 x g only a low level of enzyme activity was detected in the pellet and virtually none in the supernatant. They suggested a possible association of the enzyme with membranes. The association must be weak since gel filtration of the 3000 x g supernatant enzyme activity resulted in recovery of enzyme activity with an apparent molecular weight of 60-80,000 daltons. The anomalous behavior of yeast Δ^2 -IPP:tRNA isopentenyltransferase crude preparations on Sephadex G-100 and G-200 column and its occasional elution with nucleic acids near the void volume of these columns also indicates an association with other macromolecules.

All tRNA molecules with either $i^6\text{Ado}$ or $ms^2i^6\text{Ado}$ contain the nucleotide sequence A-A*-A- ψ -C (5,78,103,121,131) where A* is the cytokinin residue. Initial attempts by various investigators to isopentenylate oligonucleotides were unsuccessful (9,105), perhaps because of insufficient concentration of the substrates or unacceptable conformations of the oligonucleotides (56). Holtz and Klambt (56) were, however, successful in their attempt to isopentenylate oligo (A) when they used a large excess of the substrate. Oligonucleotides of lengths between 4 and 8 nucleotides could serve as substrates for the enzyme.

In some organisms, additional modifications of cytokinin residues occur after isopentenylation. One such modification is the methylthiolation of $i^6\text{Ado}$ to form $ms^2i^6\text{Ado}$ (33). E. coli tRNA contains both $ms^2i^6\text{Ado}$ and $i^6\text{Ado}$ (14,44). In E. coli, methylthiolation apparently occurs after isopentenylation (65). Supporting evidence for this pathway of $ms^2i^6\text{Ado}$ biosynthesis within tRNA is provided by Gefter and Russell (33) who showed that incompletely modified tRNA^{Tyr} suppressor

molecules contain $i^6\text{Ado}$ but not 2-methylthioadenosine. Likewise, Isham and Stulberg (59) were able to identify $i^6\text{Ado}$ in place of the usual $ms^2,i^6\text{Ado}$ in tRNA^{Phe} from methionine-starved cultures of *E. coli*. The methylthiolation process has not been extensively studied, but the precursor of the methyl group in $ms^2,i^6\text{Ado}$ was shown to be methionine by Goodman *et al.* (34). S-Adenosylmethionine serves as the intermediate methyl group carrier between methionine and $i^6\text{Ado}$ (2,32). The sulfur in $ms^2,i^6\text{Ado}$ comes from cysteine (2) and Fe^{2+} is required for the reaction (32).

Based on the small body of data available, it appears that $i^6\text{Ado}$ in tRNA also serves as the precursor of $\text{cis-}i^6\text{Ado}$ in tRNA. *In vitro* incorporation of ^{14}C -2-MVA into tRNA of rapidly growing, cytokinin-dependent tobacco callus resulted in labeling $i^6\text{Ado}$ and $\text{cis-}i^6\text{Ado}$ (83). By analogy with free cytokinin metabolism (80,81), the $\text{cis-}i^6\text{Ado}$ presumably originates from stereospecific hydroxylation of the $i^6\text{Ado}$ residue in tRNA. Presumably, $i^6\text{Ado}$ in tRNA is also the precursor of the $ms^2,i^6\text{Ado}$ residues in plant tRNA preparations.

(e) Function of cytokinins in tRNA

The function of cytokinins in tRNA is not completely resolved. In an early study by Fittler and Hall (25), selective modification of the isopentenyl side-chain of yeast tRNA^{Ser} with iodine resulted in a decreased binding to ribosome, but charging with serine was unimpaired. Similar results were obtained when $ms^2,i^6\text{Ado}$ in *E. coli* tRNA^{Phe} was modified by iodine treatment (24). Isopentenyl modification with bisulfite of *E. coli* tRNA^{Tyr} gave essentially the same results as iodine treatment (30).

A better approach to the elucidation of the function of cytokinins in tRNA was employed by Gefter and Russell (33). They succeeded in obtaining 3 forms of *E. coli* tRNA^{Tyr} , which differed from each other only by the degree of modification of the adenosine residue adjacent to the 3' end of the anticodon. These residues were adenosine, $i^6\text{Ado}$ and $ms^2,i^6\text{Ado}$. When these isoaccepting tRNA^{Tyr} species were tested for their ability to bind to ribosomes, to be charged with tyrosine, and to participate in protein synthesis, it was found that both protein synthesis

and ribosome binding activity increased with the degree of modification but there was no effect on the aminoacylation process. Therefore, it seems likely that these hypermodified bases play an important role in mRNA translation. However, Lactobacillus tRNA that was 50% deficient in i^6 Ado was shown by Litwack and Peterkofsky (74) to be indistinguishable from fully modified Lactobacillus tRNA in in vitro protein synthesis. In addition, a yeast mutant (mod 5-1) that contains less than 2% of the normal level of i^6 Ado grows just as well as non-mutant yeast strains under most conditions, although mod 5-1 homozygotes do not sporulate (71).

Hypermodified bases adjacent to the anticodon may aid in maintaining a single-stranded conformation in that region by virtue of the fact that their preferred N_1 -cis configuration interferes with the Watson-Crick base-pairing in the adjacent region (23,98,128). Another possible function for these hypermodified bases is to insure proper codon-anticodon interaction (29,50). It is also conceivable that these bases function as recognition sites for certain proteins that bind to tRNA molecules (50,114).

III. MATERIALS AND METHODS

Materials

Saccharomyces cerevisiae Strain AP 1 was obtained from Dr. Dallice Mills of the Department of Botany and Plant Pathology, Oregon State University. Fresh pig liver was from Clark Meat Lab, Oregon State University. Commercial wheat germ was processed by Centennial Mills, Portland, under the brand name of "Crown".

Materials bought from Sigma Chemical Company were amino acids, Tris-HCl, mercaptoethanol, tetradecyltrimethylammonium bromide (TTAB), Folin-Ciocalteu reagent, and the enzymes ribonuclease T₁, Crotalus adamantus venom phosphodiesterase and calf intestinal mucosa alkaline phosphatase. Bovine gamma globulin was from Bio-Rad, and Bacto-peptone and Bacto-yeast extract were from Difco Laboratories. Brewers yeast tRNA was purchased from Boehringer-Mannheim and polyethyleneimine (Polymin P) from Miles Laboratories. Sephadex G-25 and LH-20 were obtained from Pharmacia, and diethylaminoethyl cellulose (DE 23) and glass fiber discs (GF/C) from Whatman. Glycerol was ordered from Baker. New England Nuclear supplied the Omnifluor and Amersham the 1-¹⁴C- Δ^3 -IPP.

Culturing of Yeast Cells

The yeast cells were aseptically cultured in pH 6.0 YEP medium (see Table 2) at 30°C with constant shaking. The cells were harvested during late log or early stationary phase by centrifugation at 4000 x g for 10 min. Pelleted yeast cells were drained of supernatant and stored at -20°C. About 16 g (wet weight) of yeast were obtained per liter of culture.

Permanganate Treatment and Subsequent Isolation of Yeast tRNA

Yeast tRNA (5000 A₂₆₀ units) was dissolved in 50 ml of distilled water at 25°C. One-tenth volume (5.0 ml) of a 0.1% (w/v) KMnO₄ solution was added and the mixture incubated at 25°C for 15 min. At the end of that time, 5.0 ml (1/10 volume) of a 20% (w/v) potassium acetate

Table 2. Composition of YEP media

Compound	Weight/liter of media
Glucose	20 g
Bacto-peptone	20 g
Bacto-yeast extract	10 g
Adenine	1.0 mg
Leucine	1.0 mg
Methionine	1.0 mg
Uracil	1.0 mg

solution was added followed by 150 ml ($2\frac{1}{2}$ volumes) of cold 95% (v/v) ethanol. The mixture was kept at -20°C for over an hour and the precipitate collected by centrifugation ($14,500 \times g$ for 30 min), drained dry, and resuspended in 200 ml of 70% (v/v) ethanol containing 0.2 M sodium acetate at pH 7.5. The tRNA was pelleted ($14,500 \times g$ for 30 min), drained and dissolved in 40 ml ($125 A_{260}$ units/ml) of 0.1 M Tris (pH 7.5) buffer containing 0.2 M NaCl. The tRNA solution was loaded onto a DE 23 column (5 x 2.2 cm) and washed with 25 bed volumes of the Tris buffer. The tRNA was then eluted with 6 bed volumes of 0.1 M Tris-HCl (pH 7.5) buffer containing 1.0 M NaCl. Cold 95% ethanol ($2\frac{1}{2}$ volumes) was added to the tRNA eluate and the mixture kept at -20°C for over an hour. The tRNA was pelleted ($14,500 \times g$ for 30 min), drained and resuspended in the 70% (v/v) ethanol containing 0.2 M sodium acetate as above. The precipitate was collected by centrifugation at $14,500 \times g$ for 30 min, drained and dissolved in 9.0 ml of 0.1 M Tris-HCl (pH 7.5) containing 0.2 M NaCl. The tRNA sample was applied to a Sephadex G-25 column (180 ml bed volume) equilibrated with the same buffer. The tRNA-containing fractions were combined and the tRNA precipitated with $2\frac{1}{2}$ volumes of cold 95% ethanol. The precipitated tRNA was pelleted as usual, drained, and washed by suspending in 100 ml of the 70% (v/v) ethanol containing 0.2 M sodium acetate. The tRNA was then pelleted, drained and dissolved in water (about 25 ml) to give a

final concentration of ca. 150 A_{260} units/ml. This tRNA solution was stored frozen at -20°C .

Protein Assay

A modified Lowry protein assay technique (72) was employed. A protein standard curve was prepared using bovine gamma globulin in a concentration range of 27-136 $\mu\text{g}/0.1\text{ ml}$. Water (0.9 ml) was added to each of the gamma globulin samples followed by 5.0 ml of the alkaline copper solution (71). After 10 minutes at room temperature, 0.5 ml of 1 N Folin-Ciocalteu phenol reagent was added. The absorbance at 660 nm was taken 30 min later with a Hitachi Perkin-Elmer UV-Vis Spectrophotometer (Coleman 139). Samples to be assayed for protein were diluted with water to a protein concentration range of 30-80 $\mu\text{g}/0.1\text{ ml}$ and assayed in the same manner as the gamma globulin standards.

Assay for IPP Isomerase

The incubation mixture contained 100 mM Hepes pH 7.0 buffer, 2 mM MnCl_2 , 0.5 mM Mercaptoethanol, 10 mM NaF, 0.1 μCi 1- ^{14}C - Δ^3 -IPP (56 mCi/mmole) and appropriately diluted enzyme preparation in a final volume of 1.0 ml. Incubations were conducted in 15 ml clinical centrifuge tubes for 10 min at 37°C . The reaction was terminated by the addition of 0.1 ml of concentrated HCl. The acidified solution was incubated for another 30 min at 37°C followed by the addition of 0.35 g of NaCl. The mixture was vortexed for 90 sec to obtain a saturated salt solution, and then extracted twice with 3.0 ml of scintillation-grade toluene. The two phases were separated by centrifugation at top speed in a clinical centrifuge, and 1.0 ml aliquots of each toluene extract were assayed for radioactivity in a liquid scintillation spectrometer (Packard Tri-Carb Model 2405) in 10 ml of a toluene-based scintillation fluid (Omnifluor, 4 g/liter). Enzyme dilutions that gave no more than 10% conversion of Δ^3 -IPP to the Δ^2 -isomer were used for calculations of enzyme activity. One enzyme unit of IPP isomerase activity is defined as that which converts 1 μmole of Δ^3 -IPP to the Δ^2 -isomer per min under the assay conditions used.

Tests for Phosphatase Activity in IPP Isomerase Preparations

The incubation conditions were identical to those used in the IPP isomerase assay except for the omission of NaF. As in the isomerase assay, incubation was for 10 min at 37°C, and the reaction was terminated by the addition of 0.35 g of NaCl. The reaction mixtures were extracted immediately with 3 ml of toluene (without acidification). Only one extraction with toluene was performed and 1.0 ml of the toluene phase was assayed for radioactivity in 10 ml of the Omnifluor scintillation liquid.

Assay for Δ^2 -IPP:tRNA Isopentenyltransferase

The incubation mixture contained in 4.0 ml, 50 mM Tris-HCl (pH 7.5) buffer, 5 mM MgCl_2 , 10 mM mercaptoethanol, 10 mM NaF, 60 A_{260} units (unless otherwise indicated) of permanganate-treated yeast tRNA, 0.1 μCi of 1- ^{14}C - Δ^3 -IPP (56 mCi/mmol), sufficient isomerase to convert all of the Δ^3 -IPP to the Δ^2 -isomer in 10 min at 37°C, and the sample to be assayed for the enzyme. Incubation was carried out in 15 ml Corex centrifuge tubes. The reaction was terminated by the addition of 3.0 ml of buffer-saturated phenol. The reaction mixture was then vortexed for 60 sec, and the phases separated by centrifugation at 3000 x g. The aqueous phase was removed and re-extracted with another 3.0 ml of buffer-saturated phenol. The aqueous phase was transferred to a 30 ml Corex centrifuge tube. 0.4 ml (1/10 volume) of a 20% (w/v) sodium acetate (pH 6.0) solution was added, followed by 10 ml (2½ volumes) of cold ethanol to precipitate the tRNA. The preparation was kept at -20°C for at least an hour before the precipitated tRNA was pelleted at 12,000 x g for 30 min. The tRNA pellet was drained dry, dissolved in 3.0 ml of water and re-precipitated by the addition of 3.0 ml of cold 20% (w/v) trichloroacetic acid (TCA) solution. After 10 min in the cold, the precipitate was collected on a glass fiber disc by filtration under reduced pressure. The disc was washed twice with 10 ml of a cold 10% (w/v) TCA solution and finally with 10 ml of cold Hokins Reagent (0.8 ml of 10 N NaOH plus 62.5 ml of glacial acetic acid, made to 1 liter with 95% ethanol). The disc was dried and assayed for ^{14}C in 5 ml of the toluene-based Omnifluor scintillation liquid. In calculations to determine the specific activity of the transferase

enzyme, 1 unit of Δ^2 -IPP:tRNA isopentenyltransferase activity is defined as that which isopentenylates 1 μ mole of tRNA per minute under the conditions used.

Isolation of Pig Liver IPP Isomerase

Fresh pig liver was cut into small pieces and blended in 100 g batches with 200 ml of a pH 7.5 buffer containing 0.025 M KHCO_3 , 0.25 M sucrose and 0.001 M disodium ethylenediaminetetraacetic acid in a Waring blender. Blending was for 40 sec at top speed. The crude homogenate was centrifuged for 15 min at 600 x g and the resultant supernatant re-centrifuged for 20 min at 10,000 x g. Approximately 1.5-2.0 ml of final supernatant was obtained from each gram of liver. To this supernatant, 1/15 volume of a 10% (v/v) Polymin P (adjusted to pH 7.5 with NaOH) was added dropwise with stirring. The precipitate that was formed was removed by centrifugation at 22,000 x g for 15 min. The supernatant was fractionated by ammonium sulfate precipitation. The proteins precipitating between 60 to 70% saturation were collected by centrifugation at 22,000 x g for 15 min, dissolved in a minimal volume of a 0.1 M Tris-HCl buffer at pH 7.5 containing 0.01 M MgCl and 0.02 M mercaptoethanol, and desalted on a Sephadex G-25 column (25 x 1.5 cm) equilibrated with the same buffer. The protein-containing fractions were pooled and an equal volume of glycerol was added. This preparation was kept at -20°C and used as the IPP isomerase source.

Preparation of Wheat Germ IPP Isomerase

The method employed was similar to the one used for pig liver IPP isomerase. Commercial wheat germ was blended in a 0.05 M Tris-HCl buffer at pH 7.5 containing 0.005 M MgCl and 0.01 M mercaptoethanol (4 ml/g of wheat germ) for a total of 90 sec, using 30 sec bursts, in a Sorvall Omnimixer. The homogenate was centrifuged at 600 x g for 15 min and the supernatant recentrifuged for another 15 min at 10,000 x g. The final supernatant was filtered through miracloth and the pH adjusted to 7.5 with 1 N NaOH. The filtrate was subjected to a 1/15 volume 10% (v/v) Polymin P precipitation. The precipitate was pelleted (22,000 x g for 15 min) and the pellet was resuspended in $\frac{1}{2}$ the supernatant volume of homogenization buffer containing 0.3 M KCl. The KCl extract was

clarified by centrifugation and tested for isomerase activity. The supernatant from the Polymix P precipitation was subjected to sequential ammonium sulfate precipitation as indicated in the text. The precipitate from each salt cut was dissolved in homogenization buffer and de-salted on a Sephadex G-25 column in the same buffer. The protein-containing fractions were pooled and assayed for IPP isomerase activity.

Preparation of Yeast Homogenates

To each 2 g of frozen yeast in a 20 x 150 mm screw-cap tube was added 11 g of 0.45-0.50 mm acid-washed glass beads and 1.0 ml of the homogenization buffer used in wheat germ homogenization. The contents of each tube were vortexed at top speed for a total of 90 sec in 30 sec bursts. Additional homogenization buffer (1.0 ml) was added to each tube, followed by vortexing for another 10 sec. The liquid was decanted into a beaker and the tubes rinsed twice with a total of 4.0 ml of homogenization buffer. The pH of this crude homogenate was adjusted to 7.5 with 1.0 N NaOH. The homogenate was centrifuged at 20,000 x g for 15-20 min and the resultant supernatant saved. Approximately 4.5-5.0 ml of clarified homogenate were typically obtained per 2 g of frozen yeast. The pH of this crude supernatant fraction was adjusted to 7.5 and used as the starting material for the experiments described below.

Isolation of i^6 Ado from Yeast tRNA

Yeast tRNA (50 A_{260} units in 4 ml) labeled in the isopentenylation reaction was hydrolyzed to nucleosides by treatment with ribonuclease T_1 (84) followed by snake venom phosphodiesterase and calf intestinal mucosa alkaline phosphatase (39). The yeast tRNA solution was adjusted to pH 7.5 with 0.1 N NaOH and 250 units (5 units/ A_{260} unit) of ribonuclease T_1 was added. The solution was incubated for 2 hours at 35°C. A drop of toluene was added periodically to inhibit bacterial growth. The pH was checked at 30 min intervals and adjusted to 7.5 as needed. At the end of the incubation period, the pH was brought to 8.6 and 0.01 ml of 0.1 M $MgSO_4$ was added per ml of incubation volume, followed by lyophilized snake venom (0.01 mg per A_{260} unit) and alkaline phosphatase (0.01 units per A_{260} unit). The solution was incubated at 35°C for 8 hours with periodic addition of toluene and the pH adjusted to

8.6 as needed. At the end of this incubation, more MgSO_4 (0.01 ml of the 0.1 M solution per ml of incubation volume), snake venom (0.006 mg per A_{260} unit) were added. The mixture was incubated at 35°C for another 16 hours. The pH was adjusted to 8.6 and toluene added periodically. To terminate the digestion, $2\frac{1}{2}$ volumes of 95% ethanol was added and the entire mixture evaporated to dryness in vacuo in a rotary evaporator at 35°C . The solids recovered from the digestion were extracted with 2.0 ml of water-saturated ethyl acetate (6 times, 15 min each) and the pooled extracts evaporated to dryness as before. (Essentially all of the radioactivity in the tRNA was recovered in the ethyl acetate extracts.) The solids recovered from the combined ethyl acetate extracts were dissolved in 1.0 ml of 33% (v/v) ethanol and chromatographed on a Sephadex LH-20 column (0.9 x 56 cm) in 33% ethanol. One half of each column fraction was dried at 60°C in a scintillation vial and assayed for ^{14}C . The remainder of the radioactive fractions which eluted out at the same position as the $i^6\text{Ado}$ standard were combined and dried under reduced pressure at 35°C as before together with 0.2 mg of authentic unlabeled $i^6\text{Ado}$. The residue from evaporation was dissolved in 1.0 ml of water and chromatographed on a Sephadex LH-20 column (0.9 x 58 cm) in distilled water. Each fraction was dried in a scintillation vial and assayed for ^{14}C . The elution position of the $i^6\text{Ado}$ standard was detected by monitoring the absorption of the column eluate at 265 nm.

IV. RESULTS

Initial Demonstration of Δ^2 -IPP:tRNA Isopentenyltransferase Activity in a Cell-free System from Yeast

Preliminary experiments with enzyme preparations from yeast indicated that both Δ^2 -IPP:tRNA isopentenyltransferase activity and IPP isomerase activity could be precipitated from the supernatant fraction of crude homogenates by addition of 1/15 volume of 10% (v/v) Polymix P. Extraction of the Polymix P precipitated protein with buffer containing 0.3 M KCl resulted in the recovery of both enzymes in a soluble form. The results of a typical experiment in which yeast tRNA was incubated for 40 min at 37°C with an enzyme preparation obtained in this manner are shown in Table 3. It should be noted that the incubation mixture did not contain an external isomerase preparation.

To ascertain that the label incorporated into yeast tRNA from Δ^3 -IPP was actually present in i^6 Ado residues, 50 A_{260} units of $KMnO_4$ -treated yeast tRNA were isopentenylated with the Δ^2 -IPP:tRNA isopentenyltransferase preparation described above. Incubation was for 40 min at 37°C. The reaction was terminated and tRNA recovered from the incubation mixture by phenol extraction followed by ethanol precipitation. The tRNA was further purified by precipitation with TTAB from an 0.15 M NaCl solution. TTAB was removed and the tRNA converted to the sodium form by dissolving the TTAB precipitate in 1 M NaCl followed by ethanol precipitation. The precipitate was then collected by centrifugation, drained, and dissolved in 4.0 ml of water. The tRNA was hydrolyzed to its constituent nucleosides and processed according to the procedure described in Materials and Methods. Fractionation of the ethyl acetate soluble nucleosides on a Sephadex LH-20 column in 33% ethanol is shown in Figure 1. Virtually all of the radioactivity eluted at the same position as a sample of authentic i^6 Ado chromatographed on the same column. These radioactive aliquots (50%) of the fraction corresponding to i^6 Ado were pooled and co-chromatographed with unlabeled i^6 Ado on Sephadex LH-20 column in distilled water. The elution profile is shown

in Figure 2. The radioactivity eluted from the column was coincident with the peak of UV-absorption corresponding to the unlabeled $i^6\text{Ado}$. Based on similarity of behavior on Sephadex LH-20 chromatography of authentic $i^6\text{Ado}$ and the radioactivity recovered from the tRNA, it may be concluded that the Polymin P precipitation procedure was successful in isolating yeast $\Delta^2\text{-IPP:tRNA isopentenyltransferase}$.

Preparation of IPP Isomerase from Pig Liver and Wheat Germ

The recovery of IPP isomerase activity in the Polymin P pellet from the yeast enzyme preparation suggested that this reagent might also be of value in preparing the IPP isomerase from pig liver. Accordingly, the supernatant fraction from a crude pig liver homogenate was treated with 1/15 volume of 10% (v/v) Polymin P. As shown in Table 4, the isomerase activity remaining in the supernatant after Polymin P precipitation was essentially equivalent to the total activity present in supernatants obtained by high speed centrifugation ($100,000 \times g$, 2 hr) of the homogenate.

To further purify the pig liver IPP isomerase, the supernatant fraction from the Polymin P precipitation step was fractionated by ammonium sulfate precipitation. The resulting protein preparations were de-salted on a Sephadex G-25 column and tested for both isomerase and phosphatase activity. The results are shown in Table 5. The 60-70% ammonium sulfate fraction had high isomerase activity and relatively little phosphatase activity. When stored at -20°C in 50% glycerol, this supernatant showed little loss of activity after several weeks time.

To test the pig liver IPP isomerase preparation for possible contamination with $\Delta^2\text{-IPP:tRNA isopentenyltransferase}$ activity and to determine whether it contained any other components that might interfere with the transferase assay, the 60-70% ammonium sulfate fraction was tested in the transferase assay in the presence and absence of the yeast enzyme system described above. The results are shown in Table 6. No transferase activity was present in the pig liver enzyme preparation and no inhibition of the isopentenylation reaction was observed.

Pig liver IPP isomerase prepared by ammonium sulfate fractionation of the supernatant fraction obtained after Polymin P precipitation has

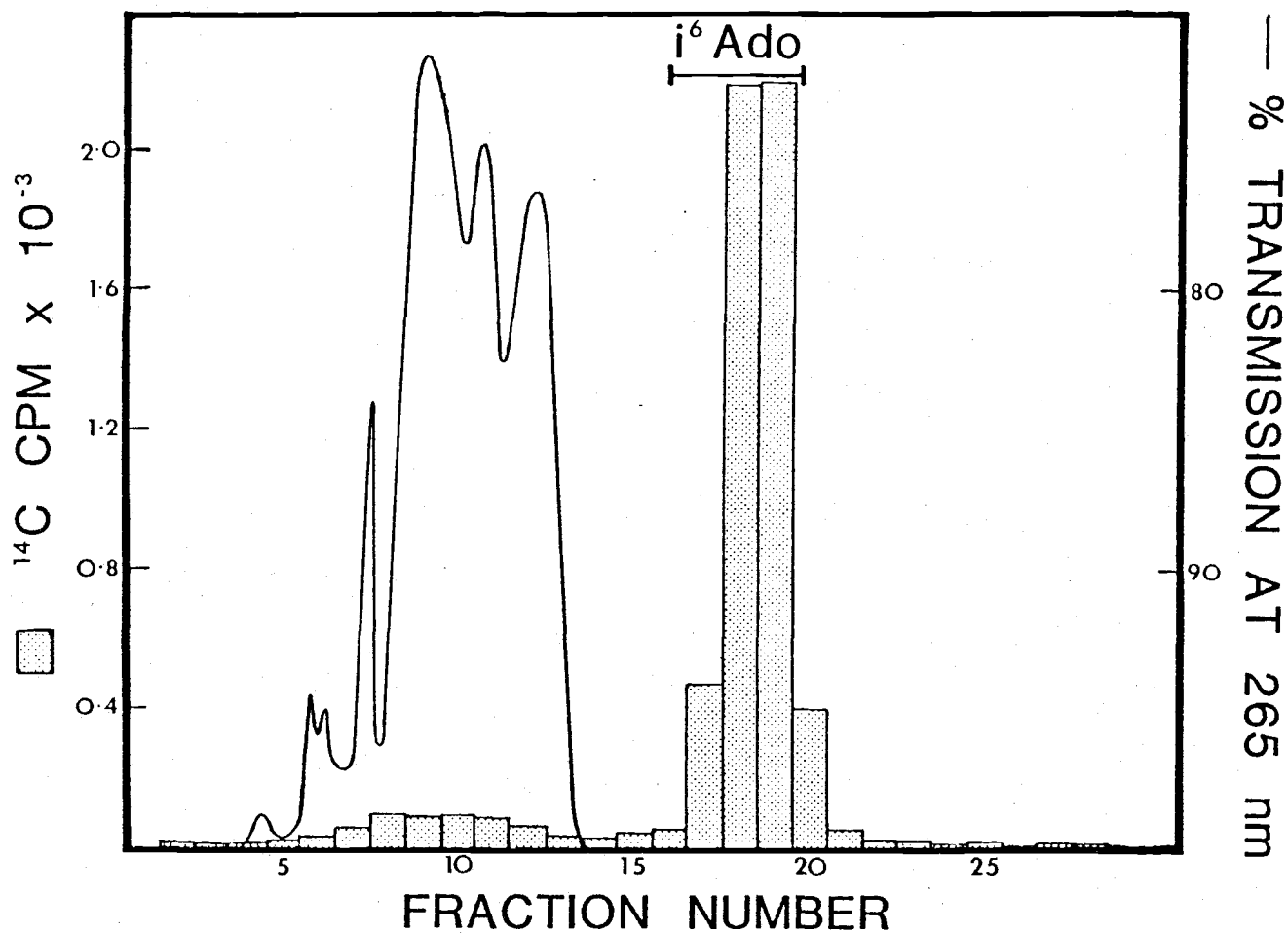


Figure 1. Sephadex LH-20 chromatography of a ^{14}C -isopentenylated yeast tRNA hydrolysate in 33% ethanol. Details of procedure are given in Materials and Methods.

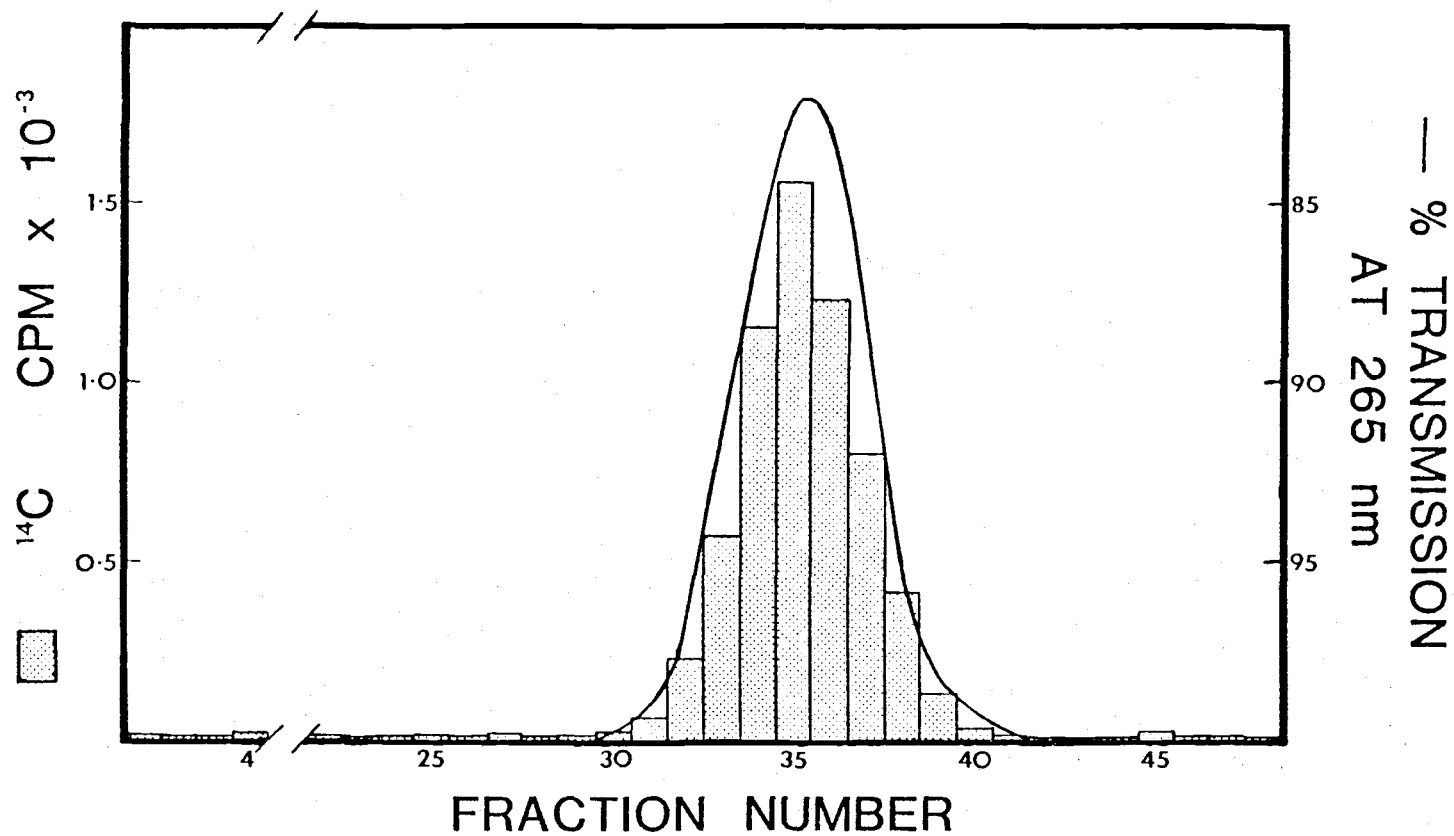


Figure 2. Co-chromatography of authentic $i^6\text{Ado}$ and radioactivity from ^{14}C -isopentenylated yeast tRNA on a Sephadex LH-20 column in distilled water. Details of procedure are described in Materials and Methods.

Table 3. Initial demonstration of yeast Δ^2 -IPP:tRNA isopentenyl-transferase activity in a cell-free preparation obtained by Polymin P fractionation.

Sample ¹	Incorporation of ^{14}C into tRNA (DPM)
KMnO ₄ -treated tRNA	9640
Untreated tRNA	60

¹The preparation assayed for the transferase activity is the protein extracted with 0.3 M KCl from a Polymin P precipitate of the low speed centrifugation crude homogenate supernatant. An aliquot was assayed with 60 A₂₆₀ units of permanganate-treated yeast tRNA and another with 60 A₂₆₀ units of untreated yeast tRNA. Details of procedure are described in Materials and Methods.

Table 4. Fractionation of pig liver IPP isomerase by ultracentrifugation and Polymin P precipitation.

Sample	IPP isomerase activity ³ (units/g liver) x 10 ⁻³
Ultracentrifugation ¹	29.9
Polymin P precipitation ²	28.7

¹The supernatant from the 10,000 x g centrifugation of the crude homogenate was centrifuged at 100,000 x g for 2 hours and the resultant supernatant fractionated with ammonium sulfate. The proteins that precipitated out between 70 to 80% (NH₄)₂SO₄ saturation were dissolved in a minimal volume of homogenization buffer and dialyzed against the same buffer. The dialyzed preparation was then assayed for enzyme activity.

²The supernatant from the 10,000 x g centrifugation of the crude homogenate was subjected to a 1/15 volume 10% (v/v) Polymin P precipitation and the clarified preparation processed by (NH₄)₂SO₄ precipitation described for the ultracentrifugation supernatant. The dialyzed preparation was then assayed for enzyme activity.

³The unit of IPP isomerase activity is defined in Materials and Methods

Table 5. Ammonium sulfate fractionation of pig liver IPP isomerase.

Sample ¹ (NH ₄) ₂ SO ₄	Units of IPP isomerase Activity ²		Units of Phosphatase Activity ³	
	per g liver	per mg protein	No NaF	10 mM NaF
50-60%	259	3.20	0.182	0.021
60-70%	68	2.10	0.019	0.006
70-80%	16	0.60	0.010	0.006

¹These preparations are the sequential ammonium sulfate saturation cuts of the supernatant after a 1/15 volume 10% (v/v) Polymix P precipitation of the pig liver homogenate. Details of procedure are given in the text.

²The unit of IPP isomerase activity is defined in Materials and Methods.

³A unit of phosphatase activity is that which dephosphorylates 1.0 μ mole of IPP per min under the assay procedure used.

Table 6. Effect of the pig liver IPP isomerase preparation on yeast Δ^2 -IPP:tRNA isopentenyltransferase activity.

Sample	Relative Δ^2 -IPP:tRNA isopentenyltransferase activity (^{14}C DPM incorporated into tRNA)	
	KMnO_4 -treated tRNA	Untreated tRNA
Yeast isopentenyltransferase ¹ preparation	25,400	440
Yeast isopentenyltransferase preparation ¹ + liver IPP isomerase ²	26,100	470
Pig liver IPP isomerase ²	430	400

¹The salt-extracted 1/15 volume 10% (v/v) Polymyxin P precipitate

²The 60-70% ammonium sulfate cut of the 1/15 volume 10% (v/v) Polymyxin P supernatant of crude pig liver homogenate. Experimental details are given in Materials and Methods.

been used as an external isomerase preparation in most of the work reported here. However, an attempt was made to develop alternative sources of isomerase activity. The IPP isomerase activity present in wheat germ was examined using the method developed in the isolation of the pig liver isomerase. The wheat germ IPP isomerase activity recovered in the supernatant of crude homogenates, in the supernatant obtained after Polymin P precipitation, and in the 0.3 M KCl extract of the Polymin P precipitated proteins are shown in Table 7. As expected from the results obtained with the pig liver enzyme, activity was not precipitated by Polymin P. Fractionation of the wheat germ Polymin P supernatant by ammonium sulfate precipitation gave the results shown in Table 8. Although isomerase activity can be obtained from wheat germ, the specific activity of the best preparation obtained here was much lower than that of the corresponding preparations from pig liver.

Properties of the IPP Isomerase from Yeast

The response of the IPP isomerase from yeast to Polymin P fractionation contrasts with that of the corresponding enzyme from pig liver and wheat germ. In the case of yeast, most of the IPP isomerase activity may be precipitated from the supernatant fraction of crude homogenates by the addition of 1/15 volume of 10% (v/v) Polymin P. This is illustrated in Table 9 in which the isomerase activity remaining in the supernatant after the addition of Polymin P is compared with the activity recovered from the precipitate by extraction with 0.3 M KCl.

The precipitation of yeast IPP isomerase by Polymin P was examined in more detail in the following experiment. The supernatant from a crude yeast homogenate was divided into 5 equal aliquots. Each aliquot was subjected to fractionation with varying concentrations of Polymin P (0-20, 20-40, 40-60, 60-80 and 80-100 μ l of 10% (v/v) Polymin P per ml of supernatant). The precipitate obtained with each Polymin P cut was collected by centrifugation and extracted in buffer containing 0.3 M KCl. The KCl extracts were assayed for protein and IPP isomerase activity. The results are presented in Figure 3. Approximately 80 to 100 μ l of 10% (v/v) Polymin P per ml was required for complete recovery of isomerase activity.

Table 7. Distribution of IPP isomerase in wheat germ fractions.

Sample	Units of IPP Isomerase Activity ⁴	
	per g wheat germ	per mg protein
Crude homogenate supernatant ¹	10.10	0.106
Polymin P supernatant ²	7.40	0.137
Polymin P pellet ³	0.73	0.084

¹The 10,000 x g supernatant of the wheat germ crude homogenate.

²The supernatant after the 1/15 volume 10% (v/v) Polymin P precipitation of the crude homogenate supernatant.

³The salt-extracted pellet of the 1/15 volume 10% (v/v) Polymin P precipitation of the crude homogenate supernatant. Details of procedure are described in Materials and Methods.

⁴The unit of IPP isomerase activity is defined in Materials and Methods.

Table 8. Ammonium sulfate fractionation of wheat germ IPP isomerase.

Sample ¹ (NH ₄) ₂ SO ₄	Units of IPP Isomerase Activity ²		Units of Phosphatase Activity ³	
	Per g wheat germ	Per mg protein	No NaF	With 10 mM NaF
0-50%	1.39	0.062	0.135	0.017
50-60%	1.61	0.206	0.005	0.002
60-70%	0.66	0.097	0.001	None
70-80%	0.06	0.022	None	None

¹These samples are the desalted sequential ammonium sulfate cuts of the supernatant after a 1/15 volume 10% (v/v) Polymix P precipitation of crude wheat germ supernatant.

²The unit of IPP isomerase activity is defined in Materials and Methods.

³The unit of phosphatase activity is stated in the footnote of Table 5.

Table 9. Fractionation of yeast IPP isomerase with Polymin P.

Sample	Units of IPP Isomerase Activity ³	
	Per g yeast	Per mg protein
Polymin P supernatant ¹	2.31	0.07
Polymin P pellet ²	10.21	1.68

¹The 1/15 volume 10% (v/v) Polymin P supernatant of yeast crude supernatant.

²The salt-extracted 1/15 volume 10% (v/v) Polymin P precipitate of yeast crude supernatant.
See text for details.

³The unit of IPP isomerase activity is defined in Materials and Methods.

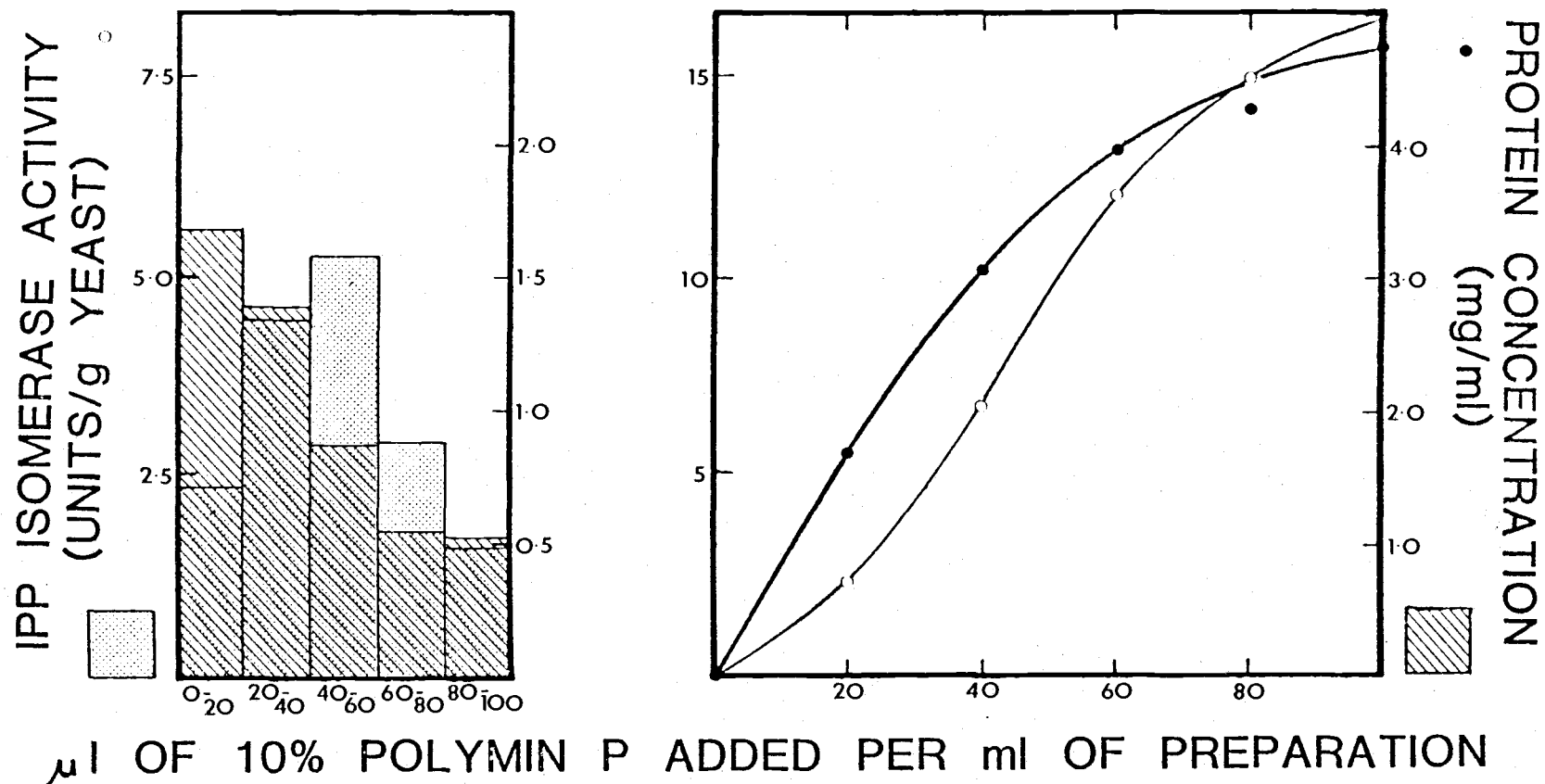


Figure 3. Precipitation of yeast IPP isomerase activity with Polymin P. (a) IPP isomerase activity and protein content in each Polymin P cut. (b) Cumulative IPP isomerase activity and protein content of preceding Polymin P cuts. Figure 3b is derived from the data presented in Figure 3a. Each data point in Figure 3b is the sum of the enzyme activity or protein content of all the Polymin P cuts below it. The unit of IPP isomerase is defined in Materials and Methods. The procedure used in this experiment is given in the text.

The efficiency of extraction of isomerase activity from the Polymin P precipitate was examined as a function of the KCl concentration in the extracting buffer. The supernatant from a crude yeast homogenate was treated with 100 μ l of 10% (v/v) Polymin P per ml. The suspension of precipitated protein was divided into 7 equal aliquots. The precipitate in each aliquot was pelleted, drained of supernatant, and extracted with buffer containing varying amounts of KCl (0 - 0.6 M). These extracts were clarified by centrifugation and assayed for protein and IPP isomerase activity. The results are shown in Figure 4. IPP isomerase activity was not solubilized at KCl concentrations below 0.1 M and essentially all the extractable activity was solubilized at 0.3 M.

Precipitation of Δ^2 -IPP:tRNA Isopentenyltransferase from Yeast

The precipitation of yeast Δ^2 -IPP:tRNA isopentenyltransferase with Polymin P was examined in the same manner as in the case of IPP isomerase. Aliquots of the crude supernatant from the yeast homogenate were fractionated by treatment with 0-20, 20-40, 40-60, 60-80 and 80-100 μ l of 10% (v/v) Polymin P per ml of supernatant. The precipitates were collected by centrifugation, extracted with buffer containing 0.3 M KCl, and the extracts assayed for protein and Δ^2 -IPP:tRNA isopentenyltransferase activity. The results are shown in Figure 5. Most of the transferase enzyme activity was precipitated from the crude supernatant by the addition of 40 μ l of 10% (v/v) Polymin P per ml supernatant.

Solubilization of yeast Δ^2 -IPP:tRNA isopentenyltransferase activity from the Polymin P precipitate was examined in a preparation obtained by the addition of 1/15 volume of 10% (v/v) Polymin P to a crude supernatant fraction. As in the case of IPP isomerase, the resulting suspension was divided into 7 equal aliquots and the precipitates collected by centrifugation. Each pellet was then extracted with buffer containing different concentrations of KCl (0 to 0.7 M). The extracts were assayed for protein and transferase activity. The results are shown in Figure 6. Transferase activity was readily solubilized at KCl concentrations above 0.2 M.

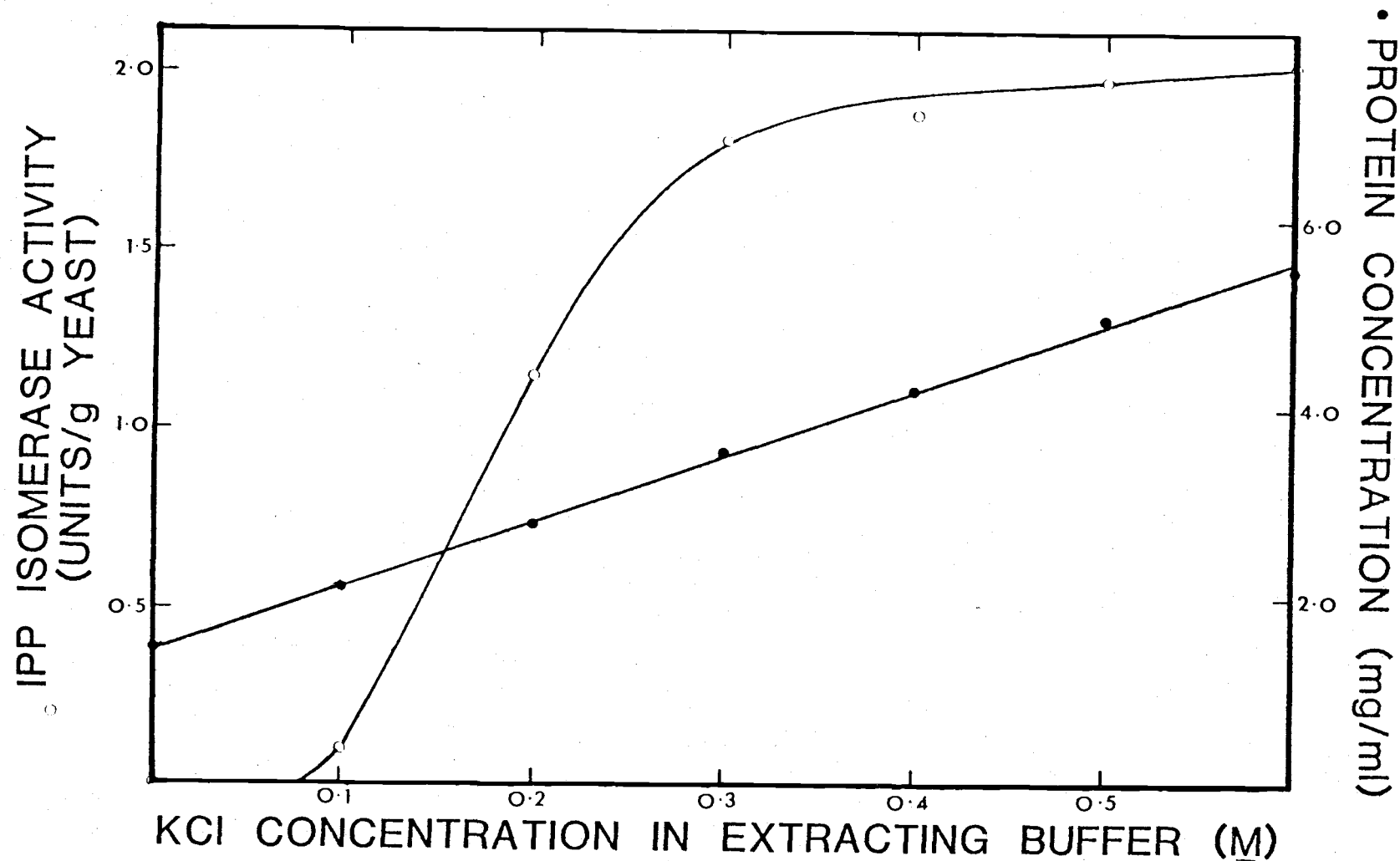


Figure 4. Solubilization of yeast IPP isomerase after Polymin P precipitation. The definition of a unit of IPP isomerase activity is given in Materials and Methods. Details of procedure are given in the text.

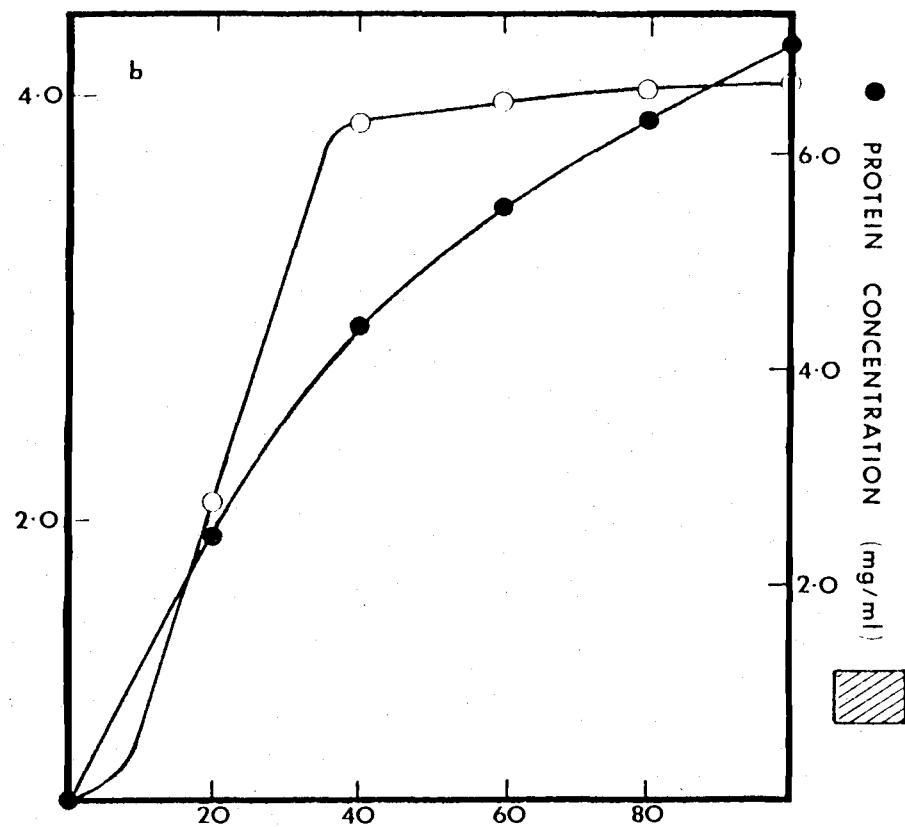
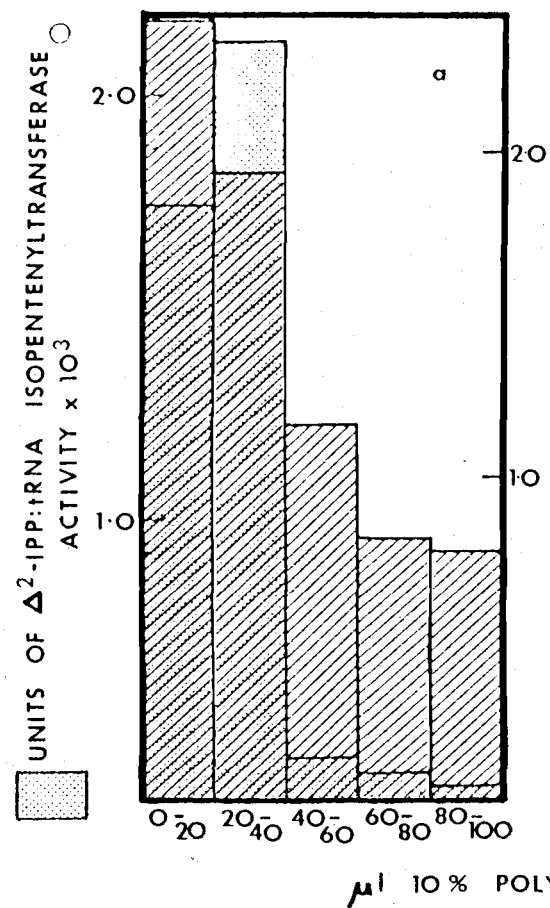


Figure 5. Precipitation of yeast Δ^2 -IPP:tRNA isopentenyltransferase activity with Polymin P. (a) Δ^2 -IPP:tRNA isopentenyltransferase activity and protein content in each Polymin P cut. (b) Cumulative Δ^2 -IPP:tRNA isopentenyltransferase activity and protein content of preceding Polymin P cuts. Figure 5b is derived from the data presented in Figure 5a. Each data point in Figure 5b is the sum of the enzyme activity or the protein content of all the Polymin P cuts below it. The unit of Δ^2 -IPP-tRNA isopentenyltransferase activity is defined in Materials and Methods. Details of procedure are described in the text.

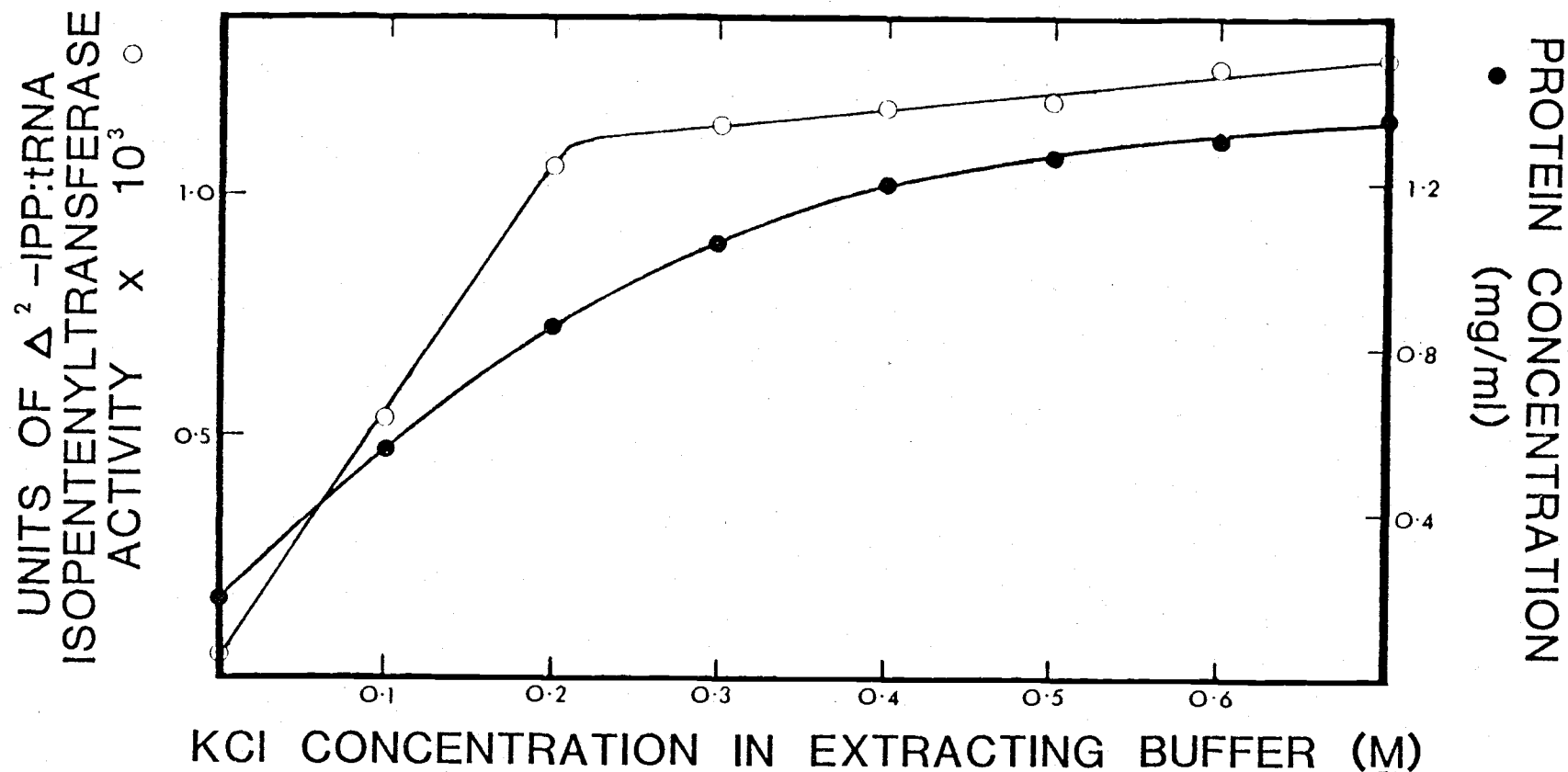


Figure 6. Solubilization of yeast Δ^2 -IPP:tRNA isopentenyltransferase from Polymin P pellet. The unit of enzyme activity is defined in Materials and Methods. The procedure used in this experiment is given in the text.

V. DISCUSSION

A Δ^2 -IPP:tRNA isopentenyltransferase preparation of relatively high specific activity was obtained from crude yeast homogenate by precipitating the enzyme with 1/15 volume of 10% (v/v) Polymin P followed by extraction of the enzyme from the precipitate using a pH 7.5 buffer containing 0.3 M KCl. The resulting enzyme preparation was essentially free of nucleic acids (60) and contained about 10% of the protein present in the crude homogenate. The IPP isomerase from yeast co-purified with the transferase activity at this stage of purification and the cell-free isopentenylation system obtained in this manner does not require the addition of an external isomerase preparation. The Polymin P procedure is suitable for use as a preliminary fractionation step in the large-scale isolation of the transferase enzyme, and it provides a simple and rapid procedure for the routine assay of transferase activity in yeast.

Polymin P has also proven useful in the preparation of IPP isomerase from pig liver. Addition of 1/15 volume of 10% (v/v) Polymin P to supernatants obtained by low speed centrifugation of pig liver homogenate gave an IPP isomerase-rich supernatant that contained about 60% of the protein present in the crude supernatant. Ammonium sulfate fractionation (60-70% saturation) of the Polymin P supernatant yielded an IPP isomerase preparation of high specific activity that was largely free of phosphatase activity. The use of the Polymin P precipitation procedure has the advantage of eliminating the time-consuming ultracentrifugation step of Holloway and Popjak (52,53), and lends itself to the large-scale preliminary purification of the isomerase enzyme from pig liver. It was also possible to obtain IPP isomerase activity from wheat germ using the pig liver Polymin P method but the level of enzyme activity recovered in the wheat germ preparation was considerably lower than that obtained from pig liver. Yeast IPP isomerase, unlike the pig liver and wheat germ enzymes, was easily precipitated by Polymin P and was readily re-solubilized by extracting the

precipitate with buffer containing 0.3 M KCl. Precipitation characteristics of yeast IPP isomerase and Δ^2 -IPP:tRNA isopentenyltransferase with Polymin P, and their solubilization behaviors from the Polymin P pellet were found to be too similar to allow the isolation of one enzyme free of the other in high yield.

Attempts to apply the Polymin P procedure to the recovery of Δ^2 -IPP:tRNA isopentenyltransferase activity from plant cells have to date been unsuccessful. In view of the difference in behavior of the IPP isomerase from yeast and wheat germ to Polymin P fractionation, it would not be surprising if the plant transferase enzyme exhibited somewhat different properties than the corresponding yeast enzyme. In any case, the procedures developed here should be useful in the investigation of the yeast enzyme, even if they are not directly applicable to other systems.

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