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Detection of dimethylallypyrophosphate : 5'amp transferase enzyme in Saccharomyces cerevisiae

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DETECTION OF DIMETHYLALLYLPYROPHOSPHATE:5'AMP TRANSFERASE ENZYME IN *Saccharomyces cerevisiae*

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CHAPTER I

RATIONALE

Although direct evidence for the de novo biosynthesis of cytokinins has been reported (Chen and Melitz, 1979; Taya et al., 1978), the actual mechanism and its control are far from being elucidated. Our lab is presently involved in studying cytokinin biosynthesis in Saccharomyces cerevisiae. This eucaryotic microbe is an excellent model system for the study of eucaryotic molecular mechanisms (Rine, 1989). S. cerevisiae contains many cellular and molecular components associated with higher eucaryotes and provides a useful model for similar phenomena in multicellular organisms (Silverman, 1987). S. cerevisiae is also quite easy to culture and its genetic system has been extensively characterized (Herskowitz, 1985). The ability to approach problems both genetically and biochemically make S. cerevisiae an exceptional model to study cytokinin biosynthesis.

Mutant strains of yeast lacking tRNA associated isopentenyladenosine (iPAdo) contain high levels of this cytokinin (Laten and Zahareas-Doktor, 1985). I propose to utilize biochemical and genetic methods to detect the putative dimethylallylpyrophosphate (DMAPP):5'-adenosine-monophosphate (AMP) transferase enzyme in Saccharomyces cerevisiae, whose existence is strongly implied by these results. Comparison of the enzymes of S. cerevisiae and

plants should lead to a better understanding of molecular mechanisms and the molecular evolution of the cytokinin biosynthetic pathway. By performing biochemical methods to detect the DMAPP:5'-AMP transferase enzyme in S. cerevisiae, I hope to provide a useful model for cytokinin study in multicellular organisms.

CHAPTER II

INTRODUCTION

Cytokinins are a class of plant hormones that regulate growth and differentiation in the tissues of higher plants. Most occur as N^6-substituted analogues of adenine. Their presence and effects have been well documented in higher plants (Letham et al., 1978a; Letham et al., 1978b; Skoog and Schmitz, 1979).

Cytokinins are known to elicit a plethora of developmental effects in higher plants. Cytokinins have been shown to promote cell division and enlargement (Letham, 1978a). They are involved in the regulation of organ development where they promote concentration-dependent root, callus, and bud formation (Skoog and Schmitz, 1979). Cytokinins also promote cell expansion in leaves, and have been implicated in the maturation of stolons (Skoog and Schmitz, 1979). Lateral buds are released from apical dominance when treated with cytokinins (Skoog and Schmitz, 1979). Moreover, cytokinins participate in flower formation, fruit formation, and trigger bud development in seeds and bulbs (Bernier, 1977; Skoog and Schmitz, 1979).

Cytokinins also have diverse metabolic effects in higher plants. Cytokinins have been shown to stimulate the respiratory rates of growing tissue (Skoog and Schmitz, 1979). Studies using labelled amino acids have shown that

cytokinins stimulate protein synthesis (Kulaeva et al., 1965), and decrease the rate of protein degradation (Takegami et al., 1977). Cytokinins activate many enzymes, including nitrate reductase and ribulose bisphosphate carboxylase (Kende, 1971; Skoog and Schmitz, 1979), while repressing many others (Higgins and Jacobsen, 1978). Furthermore, cytokinins stimulate nucleic acid synthesis and prevent degradation by suppression of nuclease activity (Skoog and Schmitz, 1979). In cultured mammalian cells, cytokinins were found to have both stimulatory and inhibitory effects on nucleic acid synthesis, depending upon the concentration added to culture media (Gallo et al., 1972; Saito et al., 1978). In human and mouse cells of normal and tumor origin, the primary effect of exogenously supplied cytokinins was inhibition of cell growth. Results have shown that RNA and protein synthesis is immediately reduced by the presence of exogenously supplied cytokinins (Burns et al., 1976).

In all monosubstituted adenines known to possess cytokinin activity, the substituents are at the N^6 position. The effect of substitution on cytokinin activity depends on the site of substitution. If the substituent is placed on the purine ring at various positions other than N^6, there is an inhibition of plant cell division (Szweykowska and Korcz, 1972). Structure-activity relationship studies of cytokinin activity of this type are analyzed utilizing a plant

bioassay. They are confined to cultured cell systems because of their high sensitivity and their reliability (Koshimizu and Iwamura, 1988). Various effects caused by the modification of the N^6 side chain have also been reported. One of the most recognizable features is that the activity varies with the length of the side chain. In tobacco callus bioassays, cytokinin activity increases with an increase in the n-alkyl chain length of the substituent, up to an optimum chain length of five carbon atoms (Skoog et al., 1967; Matsubara, 1980). Further extension of the chain reduces activity. The introduction of a double bond in the N^6 substituent significantly increases cytokinin activity. For example, the high activity of iPAdo, which has greater cytokinin effects than kinetin, the first cytokinin discovered, is due to the presence of the double bond, and not side chain branching (Skoog et al., 1967; Letham et al., 1978a). Also important in cytokinin activity is the location of the double bond (Letham et al., 1978a), and the presence of hydroxyl groups on N^6 constituents (Leonard et al., 1968) which markedly influence the activity of the cytokinin.

Along with their occurrence as plant growth factors, cytokinins have been identified as modified bases in tRNA. They are not only found in plant tRNAs, but also in the tRNAs of nearly all organisms studied, including animals and microbes (Hall and Dunn, 1975; Hall, 1971). The

modifications are restricted to the 3' end of anticodons that recognize codons beginning with uracil (Letham and Palni, 1983). The function of tRNA-associated cytokinins appears to be the enhancement of translation. They have been shown to be essential for proper binding to ribosomes in vitro (Gefter and Russel, 1969; Fittler and Hall, 1966). In mutants lacking the isopentenyladenosine [iPAdo] modification, the efficiency of translation is apparently reduced (Laten et al., 1977).

The biosynthetic pathway leading to the generation of these tRNA associated cytokinins is well documented. The isopentenyl side chain of tRNA derived cytokinins is an intermediate in the sterol biosynthetic pathway. Studies carried out on the bacterium Lactobacillus acidophilis have shown that the dimethylallylpyrophosphate (DMAPP)] side chain is formed from Δ^3 isopentenyl- pyrophosphate (Δ^3IPP) which in turn is derived from mevalonic acid (Fittler et al., 1968). DMAPP serves as the immediate donor substrate in the synthesis of tRNA derived cytokinins.

An enzyme capable of transferring the isopentenyl side chain to adenosine residues in tRNA has been studied using cell free systems. This enzyme, DMAPP:tRNA transferase, has been purified from yeast and catalyzes the transfer of the dimethylallyl group from DMAPP to the N^6 position of adenosines adjacent to the 3' side of anticodons of select tRNAs, resulting in the synthesis of N^6-(Δ^2-

isopentenyl) adenosine [iPAdo] (Rosenbaum and Gefter, 1972; Kline et al., 1969; Holtz and Klaembt, 1975; Holtz and Klaembt, 1978). Similar transferase enzymes have been purified from E. coli and L. acidophilus (Holtz and Klaembt, 1975).

Important evidence regarding the control of cytokinin biosynthesis in plant tissues comes from the recent work on Agrobacterium tumefaciens and Pseudomonas savastanoi, two similar phytopathogenic bacteria. An enzyme involved in cytokinin production (DMAPP:5'-AMP transferase) has been detected in Agrobacterium tumefaciens. This enzyme, a prenyl-transferase, transfers the same isoprenoid side chain from DMAPP to 5'-AMP to give the product isopentenyladenosine-5'-monophosphate [iPAMP]. Extensive metabolic studies indicate that iPAMP is dephosphorylated to iPAdo, and this cytokinin is converted to other cytokinin derivatives by hydroxylation of the cytokinin side chain (Letham and Palni, 1983).

The genes encoding enzymes responsible for cytokinin production in A. tumefaciens are located on tumor inducing (Ti) plasmids. A copy of the DMAPP:5'-AMP transferase gene (ipt- isopentenyltransferase) is present on the DNA fragment of the plasmid (T-DNA) that is subsequently integrated into the plant genome inducing the formation of crown gall tumors (Akiyoshi et al., 1984; Akiyoshi et al., 1983; Buchmann et al., 1985; Morris et al., 1982; Beaty et al., 1986; Akiyoshi

et al., 1985). In contrast to most cultured plant tissues, cells transformed with Ti plasmids of A. tumefaciens do not require cytokinins and auxins for cell growth and division. With the discovery that the T-DNA codes for genes directly involved in cytokinin (and auxin) biosynthesis, a molecular explanation for phytohormone independence of crown galls was provided.

Gall formation by Psuedomonas savastanoi, like that of A. tumefaciens, is generally characterized by both cell enlargement and cell division. Cytokinin production by P. savastanoi was initially discovered by Surico and co-workers in 1975 (Surico et al., 1975). Ethyl acetate extracts of culture filtrates were fractionated by thin-layer-chromatography (TLC) and analyzed by bioassay to demonstrate the presence of cytokinin species similar to iPAdo and IPAde. It has also been shown that P. savastanoi secretes a diverse number of cytokinins, including novel methylated derivatives (Morris, 1986). Recently, P. savastanoi has also been found to possess phytohormone genes that are comparable to those of A. tumefaciens in both structure and function. The genes encoding cytokinin biosynthesis in P. savastanoi have recently been investigated. Examination of cytokinin production by plasmid deletion mutants has indicated that cytokinin biosynthesis is specified by a plasmid-born gene, ptz. Cloning and expression of ptz in E. coli has shown that ptz encodes DMAPP:5'-AMP transferase

activity (Morris, 1986). Furthermore, the ptz gene has considerable homology with the ipt genes of A. tumefaciens. The overall amino acid homology is at least fifty percent and confined to the coding regions (Akiyoshi et al., 1983).

Free cytokinins are also found in a number of non-plant species. Cytokinins are involved in the development and maintenance of tumors on a variety of plants induced by bacteria and several families of insects (Elzen, 1983). Mutant strains of the yeasts S. cerevisiae and S. pombe lacking tRNA associated iPAdo contain high levels of free iPAdo (Laten and Zahareas-Doktor, 1985). Cytokinins have also been found in the cellular slime mold D. discoideum, which contains relatively high levels of iPAdo. This iPAdo serves as a precursor for the spore germination inhibitor, discadenine (Tanaka et al., 1978).

While the biosynthesis of tRNA derived cytokinins is well documented, progress towards elucidation of the free cytokinin biosynthetic pathway has been greatly retarded. Free cytokinin biosynthesis represents a minor pathway of metabolism. As are most growth factors, cytokinins are synthesized at very low levels, and the enzyme responsible for cytokinin biosynthesis is present in only trace amounts even in plants.

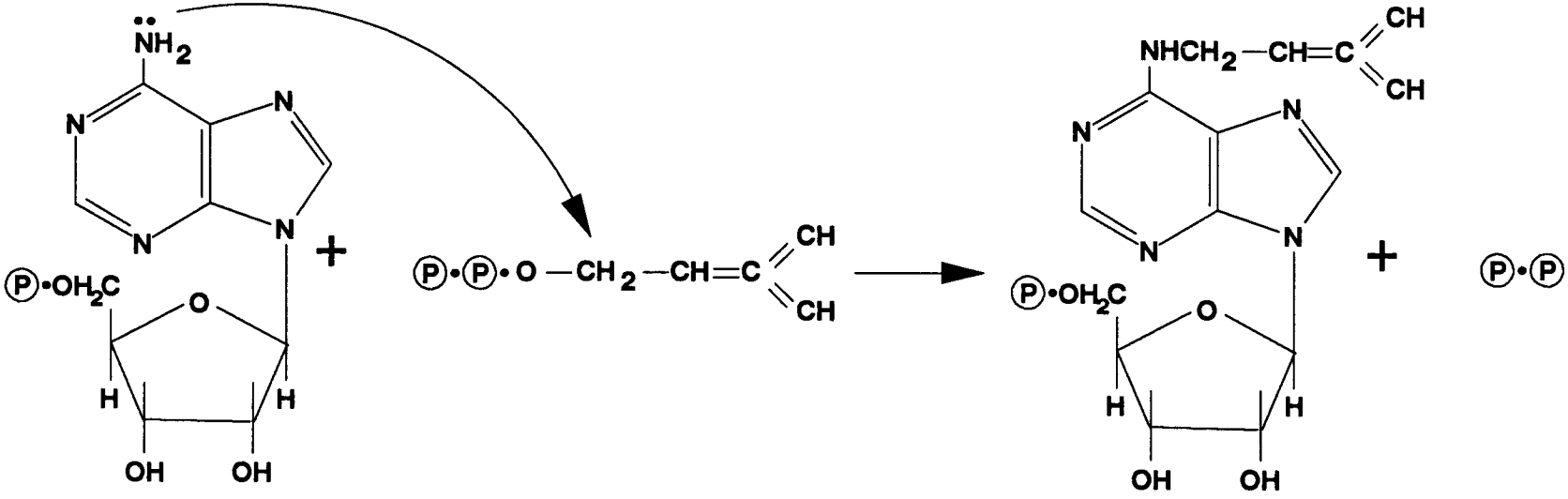
Most naturally occurring free cytokinins are N^6-isopentenyl adenine derivatives. Much attention has been focused on illuminating the biosynthetic pathway responsible

for the production of free cytokinins (Chen et al., 1976; Taya et al., 1978; Burrows, 1978; Chen and Melitz, 1979; Chen, 1982; Nishinari and Syono, 1980; Stuchbury et al., 1979; Morris, 1986; Ihara, et al., 1984).

Cell free biosynthesis of cytokinins was first demonstrated in cell-free extracts of Dictyostelium discoideum (Taya et al., 1978) and in extracts of cytokinin-independent tobacco tissue (Chen and Melitz, 1979). In the cellular slime mold D. discoideum, Taya and co-workers were able to show that the required substrates for IPAdo synthesis were 5'-AMP and DMAPP, and the reaction product was isopentenyladenosine-5'-monophosphate [iPAMP]. The synthesis can be seen in Figure 1. The reaction mixture contained ^{14}C-AMP, DMAPP, crude enzyme extract, non-dialyzed boiled cell extract in buffer at pH 7.5. After a 60 minute incubation at 27°C, the mixture was dephosphorylated, extracted with ethylacetate and subjected to TLC. Chromatograms were then scanned with a densitometer to show labelled iPAdo. The 5'-AMP was found to be an obligatory substrate and neither adenine nor adenosine were suitable substitutes.

Evidence supporting de novo biosynthesis of free cytokinins was provided by Chen in 1979 using cytokinin-independent tobacco tissue (Chen and Melitz, 1979). Using 5'- AMP and DMAPP as precursors, Chen and Melitz were able to synthesize iPAdo in a cell-free system. More

Figure 1: Synthesis of isopentenyladenosine-5'-monophosphate (iPAMP).



AMP **DMAPP** **iPAdo-5'-P**

Figure 1.

importantly, an enzyme involved in cytokinin biosynthesis was partially purified from the cytokinin autonomous tobacco callus (DMAPP:5'-AMP transferase).

Chen and coworkers were later able to further purify the DMAPP:5'-AMP transferase enzyme (Chen, 1982). The enzyme was isolated by salt precipitation, gel filtration and ion exchange chromatography. However, the degree of purification was only 48 fold compared to the crude extract. The reaction mixture contained DMAPP, ^{14}C-AMP, magnesium acetate and ß mercaptoethanol. The reaction was incubated at 37°C and the products were then analyzed by paper chromatography. It was found that DMAPP:5'-AMP transferase had an optimum pH of 7.0 and required Mg^{2+} for full activity. The molecular weight of the partially purified enzyme was estimated by gel filtration to be 52,000 ($\pm$) 2000 Da. The enriched preparation was very unstable, losing 90% of its activity after three weeks at -70°C. Substrate specificity studies performed on the enzyme showed that the enzyme is specific for AMP. When the specificity of the isopentenyl side chain was studied, it was shown that only DMAPP functioned as a substrate. A kinetic study determined that at pH 7.0 and at 37°C the K_m was 4.7 uM and the V_{max} was calculated at 81 nmol/min/mg protein.

The DMAPP:5'-AMP transferase enzyme was characterized further by Ihara and coworkers again employing D. discoideum (Ihara et al., 1984). The enzyme was purified

using salt precipitation, gel filtration, ion exchange chromatography, and affinity chromatography. These fractionation procedures showed that this enzyme was a single species and the final preparation was purified 6800 fold. The enzyme activity was measured in a reaction mixture containing DMAPP, ^{14}C-5'AMP, $MgCl_2$ and bovine serum albumen (BSA) in a buffer at pH 7.0. The reaction was run at 25°C after which bacterial alkaline phosphatase was added and the reaction further incubated. The[^{14}C]iPA was extracted with ethylacetate and radioactivity determined by liquid scintillation. It was shown that divalent metal cations profoundly affected enzymatic activity, of which 10 mM Mg^{2+} had the most profound effect. Substrate specificity studies determined that 5'-AMP was the best acceptor of the isopentenyl group, but interestingly, ADP also served as a substrate, being 60-80% as effective as 5'-AMP. The molecular mass was determined by gel filtration to be 40,000 (±) 2000 La; similar to the reported molecular mass of 52,000 (±) 2000 Da reported in tobacco callus by Chen et al. (1982). The final preparation was found to be very unstable and lost most of its activity within a day. Under the optimal assay conditions of pH 7.0 and 25°C, the K_m of the slime mold transferase enzyme was determined to be 47 times lower than the reported K_m for the tobacco enzyme (Chen, 1982).

More recently, the DMAPP:5'-AMP transferase enzyme

has been identified and characterized using a tobacco crown gall tumor line (Hommes et al., 1985). This tobacco crown gall tumor line is a relatively rich source of the DMAPP:5'AMP enzyme.

Crown gall disease is caused by the soil bacterium A. tumefaciens and is characterized by tumor growth on the affected plant. The induction and tumor maintenance is dependent upon the integration and expression of a defined segment of the DNA (T-DNA), which is derived from a large plasmid (Ti plasmid) harbored by the bacterium. Phytohormone independence in A. tumefaciens transformed cells is a characteristic due to the expression of several T-DNA genes. The genes responsible for cytokinin overproduction have been mapped to specific loci on the T-DNA (Akiyoshi, et al., 1983). It has recently been shown that the tmr gene is responsible for cytokinin overproduction by encoding DMAPP:5'-AMP transferase (Akiyoshi et al., 1984; Akiyoshi et al., 1983; Barry et al., 1984; Beaty et al., 1986; Akiyoshi et al., 1985). Thus, the high levels of cytokinins found in transformed tissue were due to the tmr gene (integrated during infection), which coded for DMAPP:5'-AMP transferase. Hommes and coworkers have reported an assay method for DMAPP:5'-AMP transferase and protocol for isolation and partial purification of the enzyme using a tobacco crown gall tumor line. The enzyme was purified using salt precipitation and

ion-exchange chromatography. These purification procedures yielded an enzyme whose activity was stable for approximately two months. Enzyme activity was measured in a reaction mixture containing DMAPP, 5'-[^{3}H]AMP, KF, $MgCl_2$, and glycerol in a pH 7.0 buffer. The reaction mixture was incubated at 25°C for 1-4 hours, and then applied to a short octadecylsilica column. Reaction products were then eluted with methanol and dried in vacuo. The dried sample was then dephosphorylated by the addition of acid phosphatase. The iPAdo was then purified on an immunoaffinity column. The reaction products were then applied to a HPLC column and the radioactivity of the iPAdo fraction was finally determined by liquid scintillation. The properties of the crown gall tobacco tissue DMAPP:5'-AMP transferase were similar to those from the enzyme isolated from tobacco callus by Chen et al., 1982.

Since these initial findings, the DMAPP:5'-AMP transferase has been further purified and characterized (Akiyoshi et al., 1984; Barry et al., 1984), and a detailed review of cell-free biosynthesis of cytokinins has been published (Letham and Palni, 1983). A comparison of enzyme purification procedures and reaction conditions can be seen in Figures 2 and 3.

Our lab is presently involved in studying cytokinin biosynthesis and metabolism in Saccharomyces cerevisiae. This eucaryotic microbe is an excellent model system for the

Figure 2: Enzyme Purification Procedures.

Figure 3: Reaction conditions.

Figure 2. Enzyme Purification Procedures

Ref.	Source	Cell Breakage	Strep. Sulfate ppt.	Amm. Sulfate ppt. (% saturatn)	DEAE Chrom.	Phosphocellulose P11 Chromatog.	Gel Filtration	Affinity Chromatog.	Fold Purificatn
I	Slime Mold	Mincing with AlO_2	X	40-70	X	X	X	X	6800X
II	Crown Gall	Blade Homog.	---	25-80	X	---	---	X	---
III	Cloned	Sonicatn	---	85	X	---	---	---	---
IV	Tobacco	Blade Homog.	---	90	X	---	X	---	48X

References: (I) Ihara et al., 1984; (II) Hommes, et al., 1985; (III) Akiyoshi, et al., 1984; (IV) Chen, 1982.

Figure 3. Reaction Conditions

Ref.	Source	Tris (mM;pH)	Hepes (mM;pH)	Mg^{2+} (mM)	K^+ (mM)	KF (mM)	Temp (°C)	Incubation Time (min)	Reaction Volume (ml.)
I	Slime Mold	20;7.5	---	5	---	---	25	30	0.1
II	Crown Gall	---	30;7.0	20	---	5	25	60-240	6.0
III	Cloned	---	120;7.3	15	160	7	22	30	8.0
IV	Tobacco	50;7.0	---	30	---	---	37	30	0.1

References: (I) Ihara, et al., 1984; (II) Hommes, et al., 1985; (III) Akiyoshi, et al, 1984; (IV) Chen, 1982.

study of eucaryotic molecular mechanisms (Rine, 1989). S. cerevisiae contains many cellular and molecular components associated with higher eucaryotes and provides a useful model for similar phenomena in multicellular organisms (Silverman, 1987). S. cerevisiae is also quite easy to culture and its genetic system has been extensively characterized (Herskowitz, 1985). The ability to approach problems both genetically and biochemically makes S. cerevisiae an exceptional model to study cytokinin biosynthesis. Mutant strains of yeast lacking tRNA associated iPA contain high levels of this cytokinin (Laten and Zahareas-Doktor, 1985). I propose to utilize biochemical methods to detect the putative DMAPP:5'-AMP transferase enzyme in Saccharomyces cerevisiae, whose existence is strongly implied by these results.

CHAPTER III

MATERIALS AND METHODS

Growth of Cells

Three strains of Saccharomyces cerevisiae were utilized, 1460, 1480 and 370 (wild type). Genotypes are as follows:

370: a

1460: a mod5 ade2 his5 lys1 trp5 can1 leu1

1480: α mod5 ade2 his5 lys1 trp5 can1 leu1

The cells were grown at 30°C in an incubating shaker to mid-logarithmic phase in liquid YPD medium (1% yeast extract, 2% peptone, 2% dextrose), and then harvested by centrifugation at 4000 x g for 20 minutes. Cells were stored at -20°C.

Cell Breakage

Disruption of the Cell Pellet with Glass Beads- (All manipulations were performed between 0-4°C)

Two volumes of modified lysis buffer (50mM Hepes, 20mM KCl, 10mM $MgCl_2$, 0.5mM dithiothreitol (DTT), 1mM phenylmethylsulfonylfluoride (PMSF), 5mM EDTA, 20% glycerol, pH 7.0) or modified lysis buffer containing 0.1% Triton X 100 (Gekko and Timasheff, 1981; Furth, 1980) were added to

one volume thawed yeast paste. Disruption of the cell pellet was performed with silanized glass beads using a Bead-Beater (Biospec Products). Fifteen one minute cycles, separated by 2 minute pauses, produced 90-95% breakage as determined by visual inspection under 400x magnification. The crude extracts were then centrifuged at 20,000xg for 20 minutes to remove debris. Protein concentrations were determined by bicinchoninic acid protein assay (B.C.A. Protein Assay, Sigma Chemical Co.) according to manufacturer's instructions.

Toluene Autolysis - (All steps were performed at 0-4°C.) Two volumes ice cold toluene, saturated with modified lysis buffer, were added to frozen yeast paste and allowed to soften. The suspension was then frozen (-78°C) in a dry ice/ethanol bath for two hours. The excess toluene was then poured off and the frozen paste was thawed overnight at 4°C. A solution containing 2M Tris, 1mM PMSF pH 8.0 was then added to the suspension (one ml per 25 g yeast paste). The suspension was centrifuged at 30,000xg for 20 minutes and the supernatant decanted and recentrifuged at 30,000xg. This was repeated a third time to remove cell debris. This extract is referred to as crude extract. Protein concentration was determined as described above.

Protease Activity

Since phenymethylsulfonylfluoride (PMSF) alone is often insufficient to protect proteins from degradation during isolation and purification (Jones, 1983; Gordon, 1977), leupeptin (0.5 μg/ml; Umezawa, 1976), and pepstatin (0.7 μg/ml) were added to all lysis buffers to improve the efficacy of protease inhibition. To test for proteolytic degradation and to verify the effectiveness of protease inhibition, an endoprotease test for detection and determination of endoproteolytic activity (Boehringer Mannheim Biochemicals Endoprotease Test Kit) was performed after each purification step according to manufacturer's instructions.

tRNA Isolation

Approximately 10g (packed wet weight) of each strain were used. Ten ml of cold TME buffer (0.05 M Tris-HCl, 0.01 M $MgCl_2$, 0.1 M EDTA, pH 7.5) and 10 ml TME-saturated phenol were added to the cells and the suspension was agitated in the cold (4°C) for one hour. The suspension was centrifuged at 10,000xg for 10 minutes to separate phases. The aqueous phase was decanted and combined with an additional 10 ml TME-saturated phenol while the phenol phase was combined with an additional 10 ml of TME buffer. The phases were agitated for one hour as before and the aqueous phases were combined. NaCl was added to a final concentration of 0.2 M. After mixing, 2.2 volumes of ethanol were added and the

samples were placed at -20°C overnight. The samples were then centrifuged at 15,000xg for ten minutes, and the pellet was washed with cold ether. The pellet was recentrifuged, and dried *in vacuo*. The pellet was dissolved in 10 ml T.1 S buffer (0.05 M Tris-HCl, 0.01 M $MgCl_2$, 0.1 M NaCl, pH 7.5) and loaded onto a DEAE cellulose column pre-equilibrated with T.1S buffer. The absorption of the column eluate was monitored at 254nm and all fractions having an absorbance greater than 0.5 were combined. Next, 2.2 volumes of ethanol were added and placed at -20°C overnight. The sample was centrifuged for 10 minutes at 15,000xg and the supernatant was discarded. The pellet was then washed with cold ether and recentrifuged. The supernatant was discarded and the pellet dried *in vacuo*.

Digestion of tRNA

Transfer RNA was enzymatically digested to nucleosides. The reaction mixture contained per μl: 1 μg tRNA, 0.2 μg pancreatic ribonuclease, 0.2 μg snake venom phosphodiesterase, 0.2 μg bacterial alkaline phosphatase, 10 nanomoles NaCl, 30 nanomoles sodium bicine. Reactions were incubated at 37°C for six hours and frozen at -20°C overnight. Samples were thawed by briefly heating to 37°C, and diluted three-fold with water prior to radioimmunoassay analysis.

Radioimmunoassay (RIA)

The method employed by Milstone et al. (1978) was originally used with minor modifications. The RIA is a competitive assay in which antigen in the sample competes with ^{3}[H]iPAdo trialcohol for binding sites on the antibody. Monoclonal anti-iPAdo antibodies were a generous gift from Dr. Roy Morris. All filtration was accomplished using a Millipore sampling manifold.

Reaction mixtures contained: 25 µl 10X TS buffer (0.1 M Tris, 1.4 M NaCl, pH 7.4), 50 µl TS buffer containing 1% ovalbumin (w/v), ^{3}[H]iPAdo trialcohol (approx. 2500 cpm), 50 µl sample or iPAdo standard, 5 µl anti-iPAdo antibody, and water to a final volume of 245 µl.

The reaction samples were incubated at room temperature for 30 minutes, and then placed on ice for an additional 30 minutes. Membrane filters (Metricel GN-6, 25 mm) were wetted with 2 ml of ice cold TS buffer approximately two minutes before the end of the cooling step. Samples were quickly filtered through membranes, and each filter was then washed three times with 2 ml of ice cold TS buffer. Filters were then dried under an incandescent light for 30 minutes, transferred to scintillation vials containing 5 ml scintillation cocktail (Ecolume), and counted on a Packard Tri-Carb liquid scintillation counter.

Preparation of Nucleic Acid-free Protein Fractions

(All procedures were performed at 0-4°C.)

Streptomycin sulfate precipitation - For removal of ribosomes and associated RNA, the crude enzyme preparation was mixed with 1/2 volume of a 10% streptomycin sulfate solution. The mixture was then centrifuged for 30 minutes at 15,000xg and the supernatant was saved for ammonium sulfate fractionation.

Ammonium sulfate fractionation - Solid ammonium sulfate was added to the supernatant and the precipitate obtained between 20 and 80% saturation was extensively dialyzed against 50 mM Hepes, 20 mM $MgCl_2$ and 10 mM KCl (buffer #1). The appropriate protease inhibitors (leupeptin, pepstatin, PMSF) against endo-, carboxy- and aminopeptidases were added to all dialysis buffers to prevent protein degradation (Jones, 1983). Protein concentrations were determined by BCA protein assay. Nucleic acid-free protein fraction aliquots (referred to as crude homogenate) were stored at -70°C.

Ion-Exchange Chromatography- Ion-exchange chromatography was performed according to Hommes et. al. with minor modifications (Hommes et al., 1985; Eberhardt and Rilling, 1975; Rilling, 1985; Pharmacia, 1981). DEAE-cellulose (Sigma Chemical Co.) was precycled according to manufacturer's instructions, washed with water and suspended in 10 volumes of 1 M Tris, pH 7.0. The pH of the stirred

suspension was adjusted to pH 7.0 with 1 M HCl. The cellulose was then washed with 100 volumes of 50 mM Tris, pH 7.0, followed by 20 volumes 50 mM Tris, 20 mM KCl, 10 mM $MgCl_2$, 20% v/v glycerol, pH 7.0 (Buffer A). A column was then poured and equilibrated with 2.5 volumes Buffer A. The dialyzed ammonium sulfate fraction was then loaded onto the column at 0.5 ml/min and washed with 3 volumes Buffer A. The putative DMAPP:5'-AMP transferase enzyme was finally eluted with Buffer A containing 0.3 M KCL. All fractions were then stored at -70°C.

Ultrafiltration - (performed at 0-4°C) Selected fractions were collected and reduced in volume by ultrafiltration with an Amicon CM 10 membrane (molecular weight cutoff: 5,000) under nitrogen pressure (60 psi). All fractions were stored at -70°.

Synthesis of DMAPP

The first procedure to synthesize allelic pyrophosphates was devised in 1959 by Cramer and Bohm (Cramer and Bohm, 1959). Since then, Poulter and colleagues have introduced a procedure for the synthesis of DMAPP via a direct displacement reaction (Davisson et al., 1985; Davisson et al., 1986). As this procedure is easier to execute and product yields are higher than in previous reports (Cornforth and Popjak, 1969), this method was used for the synthesis of DMAPP. Synthesis is shown in Figure 4.

Figure 4: Synthesis of DMAPP.

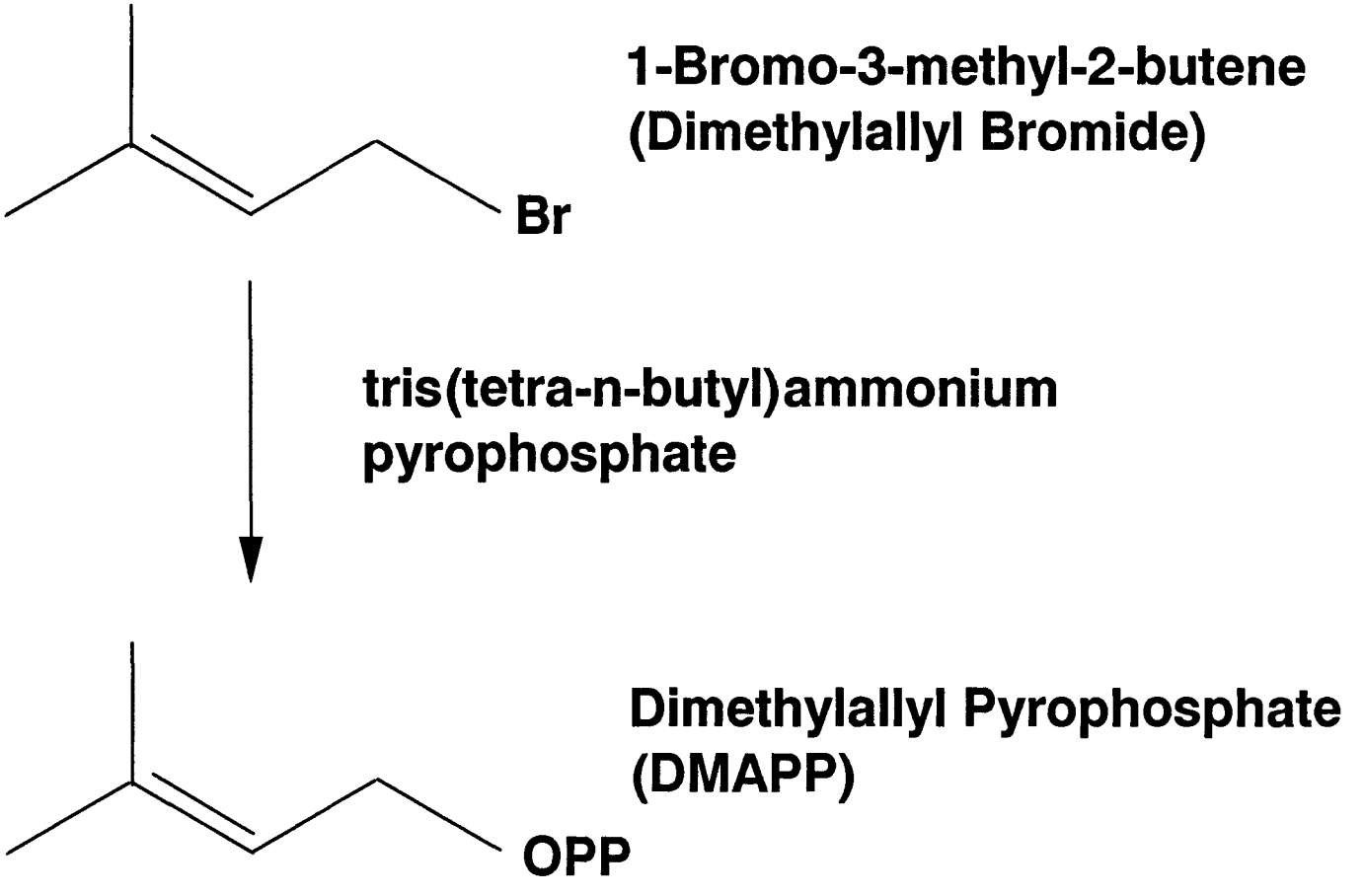

1-Bromo-3-methyl-2-butene
(Dimethylallyl Bromide)
Br
tris(tetra-n-butyl)ammonium
pyrophosphate
Dimethylallyl Pyrophosphate
(DMAPP)
OPP

Figure 4.

Reagents - 1-Bromo-3 methyl-2 butene (dimethylallyl bromide) was purchased from Lancaster Chemical Company. Tris-(tetra-n-butylammonium) hydrogen pyrophosphate was synthesized from disodium dihydrogen pyrophosphate (Stauffer Chemical Company) and tetra-n-butylammonium hydroxide (Aldrich Chemical Company). All other chemicals were lab grade.

Procedure - Tris (tetra-n-butyl) ammonium hydrogen pyrophosphate (2.27g; 2.52 mmol) was dissolved in 4.5 ml acetonitrile. Next, dimethylallyl bromide, (179 mg; 1.20 mmol) was dissolved in 0.5 ml acetonitrile, and added to this solution. The reaction mixture was stirred at 25°C for 2 hours before the solvent was removed by rotary evaporation. The opaque residue was then dissolved in 3.0 ml of 1:49 (v/v) isopropanol: 25 mM ammonium bicarbonate and passed through a pre-equilibrated Dowex AG 50W-X8 cation exchange column. The column was eluted with 2 column volumes of 1:49 (v/v) isopropanol: 25 mM ammonium bicarbonate and the eluate was lyophilized to dryness to yield 780 mg of impure DMAPP.

DMAPP Purification - After ion exchange and lyophilization, the residue consisted of a mixture of ammonium salts of organic diphosphate, inorganic pyrophosphate and the displaced leaving group (Davisson et al., 1985; Davisson et al., 1986). The white solid obtained

from cation exchange was dissolved in 4 ml of 0.1 M ammonium bicarbonate. Ten milliliters of a 1:1 (v/v) mixture of isopropanol:acetonitrile were added. A white precipitate formed after 1-2 minutes of vortexing. The suspension was cleared by centrifugation (5 min at 5000xg). The pellet was then removed and the process was repeated 3 times. The combined supernatants were concentrated by rotary evaporation and then freeze-dried. The resulting white solid was then collected and stored at -78°C.

DMAPP Identification - Thin layer chromatography was used to identify DMAPP. DMAPP samples were loaded onto 0.1 mm cellulose thin layer chromatography plates (Macherey-Nagel and Co.) and developed (8 cm) with isopropanol: acetonitrile: 0.1 M ammonium bicarbonate 4.5:2.5:3 (v/v/v). The plates were sprayed with 1% sulfosalicyclic acid in 3:2 (v/v) ethanol:water, air dried, and sprayed with 0.2% ferric chloride in 4:1 (v/v) ethanol:water. Pyrophosphate-containing material appeared as white spots on a pink background.

NMR of reagents and DMAPP - Samples of the pyrophosphate salt (tris (tetra-n-butylammonium) hydrogen pyrophosphate) and dimethylallyl bromide (1-bromo-3-methyl-2-butene) were then prepared for ^{1}H NMR just prior to analysis with deuterium oxide. DMAPP was prepared for ^{1}H NMR and ^{31}P NMR with deuterium oxide adjusted to pH 8.0 with ammonium deuteroxide-d_5 (Aldrich Chem. Co.). All samples

were prepared in concentration of 35-50 mg/ml with tetra methyl silane as an internal reference. ^{31}P NMR spectra used external phosphoric acid as an internal reference.

Positive Control

E. coli strain HB101 (pTHH21) was employed. (This strain was a generous gift from Dr. Stephen Farrand.) This strain contains a cosmid clone of pTiC18, which contains the ipt region. The plasmid vector encodes resistance to tetracycline (Tc) (Hayman and Farrand, 1988). Cells were grown to late logarithmic phase in L-medium (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.5% NaCl, 10mM Tris, 1mM $MgSO_4$, pH 7.5) containing 15 μg/ml Tc and 20 μg/ml acetosyringone; used to induce expression of the ipt gene (Rogowsky et al., 1987). An assay and partial purification of the DMAPP:5'-AMP transferase was then performed according to Hommes et al., 1985.

DMAPP:5'-AMP Transferase Assay

The assay parameters of Hommes et al., 1985 were employed with minor modifications. All reactions were run in silanized glassware or polypropylene containers, since cytokinins have a high affinity for untreated glass surfaces.

Assay - The assay mixture contained: 50 mM Hepes, 20 mM $MgCl_2$, 10 mM KCl, 0.15 mM DMAPP, 10 μCi 5'-[2-^{3}H]AMP,

10 μM 5'-AMP, and crude homogenate containing 75 mg of protein, pH 7.0. The assay mixture was vortexed and incubated at 30°C. One hundred ng cold iPA was added as carrier. The reaction was then stopped by heating to 65°C for 1 minute. The reaction mixture was then cooled to 37°C for 5 minutes prior to dephosphorylation.

Dephosphorylation - The initial product of the transferase reaction was expected to be iPAMP. The iPAMP was dephosphorylated to iPA by adding 20 units bacterial alkaline phosphatase (BAP) in one ml of 0.5 M Tris HCl, pH 8.0.

The reaction was incubated for 1 hour at 37°C and was spun (1500xg for 1 min) in a clinical centrifuge at 1600 rpm to remove any precipitated debris. The mixture was then eluted through a short octadecylsilica (ODS) column (Sep Pac, Waters Assoc.) to remove non-isopentenylated products such as adenosine. The labelled isopentenylated products were then eluted with methanol.

High Performance Liquid Chromatography

A slight modification of published procedures (Anderson and Kempt, 1979; Hartwick and Brown, 1977; Wakizaka et al., 1979) was used to analyze labelled iPA present in the reaction products. To resolve the radiolabelled products of the cell-free extracts, samples were run on a reverse phase ODS C_{18} column (Brownlee) using

the following gradient program (Macintosh McRabbit Automated Gradient Programmer; flow rate, 1 ml/min): 5%-20% methanol over 20 min., 20%-80% over 20 min. The sample was then dissolved in 100 ul 95% methanol and applied to the column. Fractions were then collected (1.0 min) and their radioactivity determined by liquid scintillation. Radioactive peaks were evaluated against the retention times of authentic cytokinins.

Reverse Phase High Performance Thin Layer Chromatography

Thin layer chromatography was also used to analyze total reaction products. Samples were loaded onto a reverse phase C_{18} thin layer chromatography plate (Whatman LKC_{18}) and developed (15 cm) with ethanol:0.5 M NaCl (Sherman, 1981). Plates were then sprayed with 7% 2,5-diphenyloxazole (PPO) in ether, dried, placed on a sheet of Kodak X-OMAT R film, and exposed at -76°C for 5 days (Bartlesman et al., 1983; Bonner and Laskey, 1974). The identity of labelled reaction products was then determined by comparison against the elution patterns of authentic cytokinins.

CHAPTER IV

RESULTS

Radioimmunoassays

The RIA standard curve was generated as described in Materials and Methods and contained 5 μl anti-iPAdo antibody, 6.67 μl ^{3}[H]iPAdo, and known concentrations of unlabelled iPAdo ranging from 50 nmol to 200 nmol in 0.25 ml 50 mM Tris, 10 mM $MgCl_2$, pH 7.5. The results are summarized in Figure 5 with each point representing 5 runs (linearity from 15-40% inhibition and from 80-85% inhibition not shown).

iPAdo levels of the tRNA samples (wild type, strains 1460 and 1480) that were isolated and digested to nucleosides were measured by comparing binding inhibitions with the standard curve. A 1:20 dilution with 50 mM TRIS, 10 mM $MgCl_2$ was performed on all samples to bring the concentration of iPAdo into the established range of the standard curve.

The iPAdo concentrations of the tRNA samples were calculated from the standard curve and converted to pmoles/A_{260} tRNA. The results of the RIA's for tRNA- derived iPAdo are shown in Table 1.

Figure 5: iPAdo standard inhibition curve.

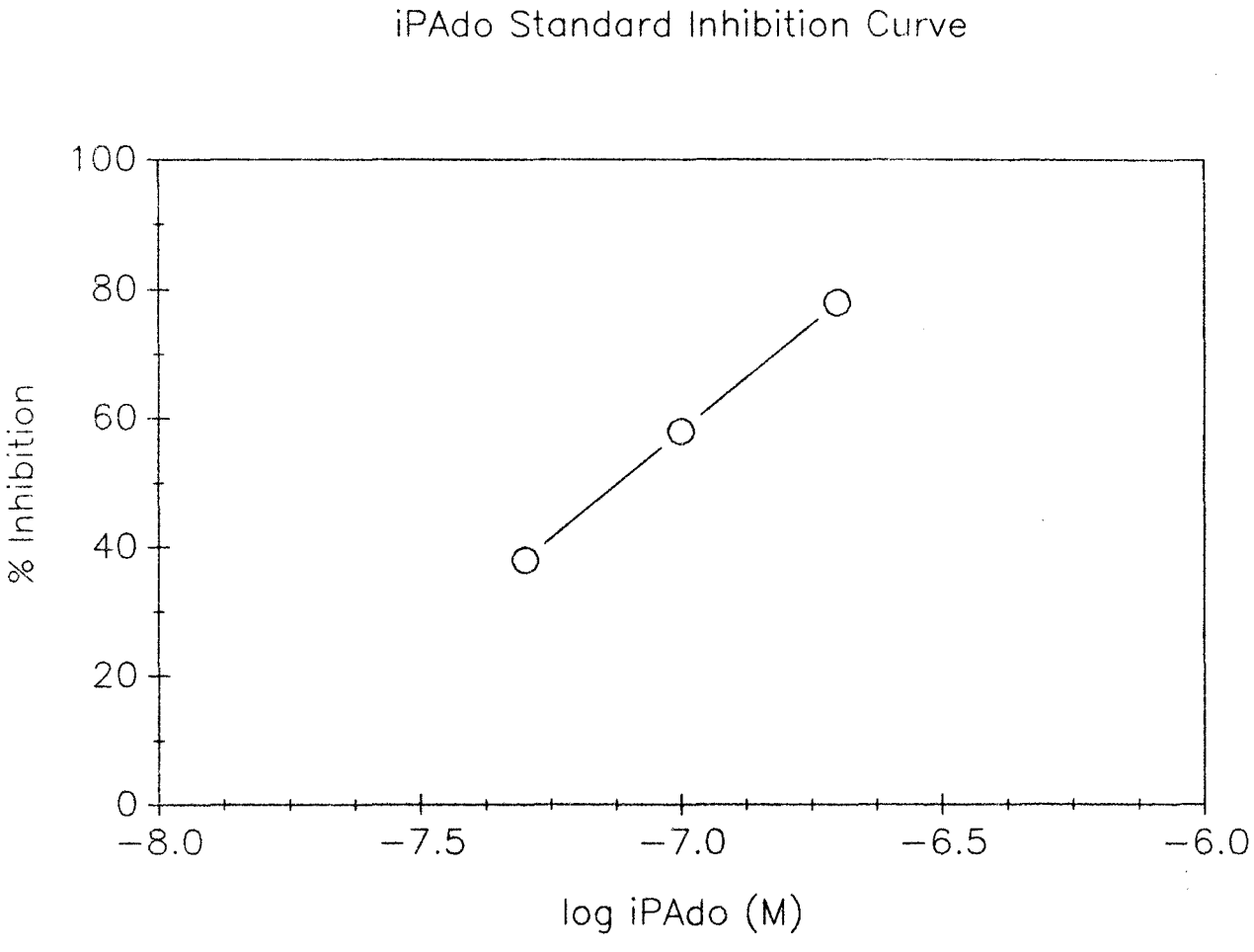

iPAdo Standard Inhibition Curve
100
80
60
40
20
0
% Inhibition
−8.0
−7.5
−7.0
−6.5
−6.0
log iPAdo (M)

Figure 5.

	STRAINS		
	370 (wild type)	**1460** (mod5)	**1480** (mod5)
cpm	340	252	428
% inhibition	23	16	28
dilution	1:20	1:40	1:40
[iPAdo] M	3.55×10^{-8}	7.50×10^{-8}	1.78×10^{-8}
pmol/ A_{260} tRNA	71.2	7.48	1.78

Table 1: tRNA associated iPAdo in wild-type and isopentenylated tRNA deficient yeast mutants.

Mutants carrying the mod5 mutation have reduced DMAPP:tRNA transferase activity (Laten et al., 1985), thus reducing a major competitor for substrate in the DMAPP:5'-AMP transferase assay and possible contributing enzyme products. The calculated values of iPAdo correspond with previously reported values for tRNA-associated iPAdo (Laten and Zahareas-Doktor, 1985). A comparison of the tRNA-derived iPAdo from S. cerevisiae shows that the wild-type strain contains almost ten times as much iPAdo in its tRNA when compared with strain 1460. In addition, the wild type strain contains forty times as much iPAdo in its tRNA than the mod5 strain 1480. Since S. cerevisiae strain 1480 had the lowest concentration of iPAdo in its tRNA, isolation of DMAPP:5'-AMP transferase from this strain was attempted.

DMAPP Synthesis

Thin layer chromatography was used to identify DMAPP. 10 μg DMAPP were dissolved in development buffer, developed by cellulose thin-layer chromatography, and visualized. The solvent system was selected to ensure that ammonium phosphate and ammonium pyrophosphate would have lower R_f values than the desired organic phosphate (Davisson et al., 1985; Davisson et al., 1986). The product synthesized had an R_f value of 0.34 (results not shown). This is very close to the published value of R_f 0.35

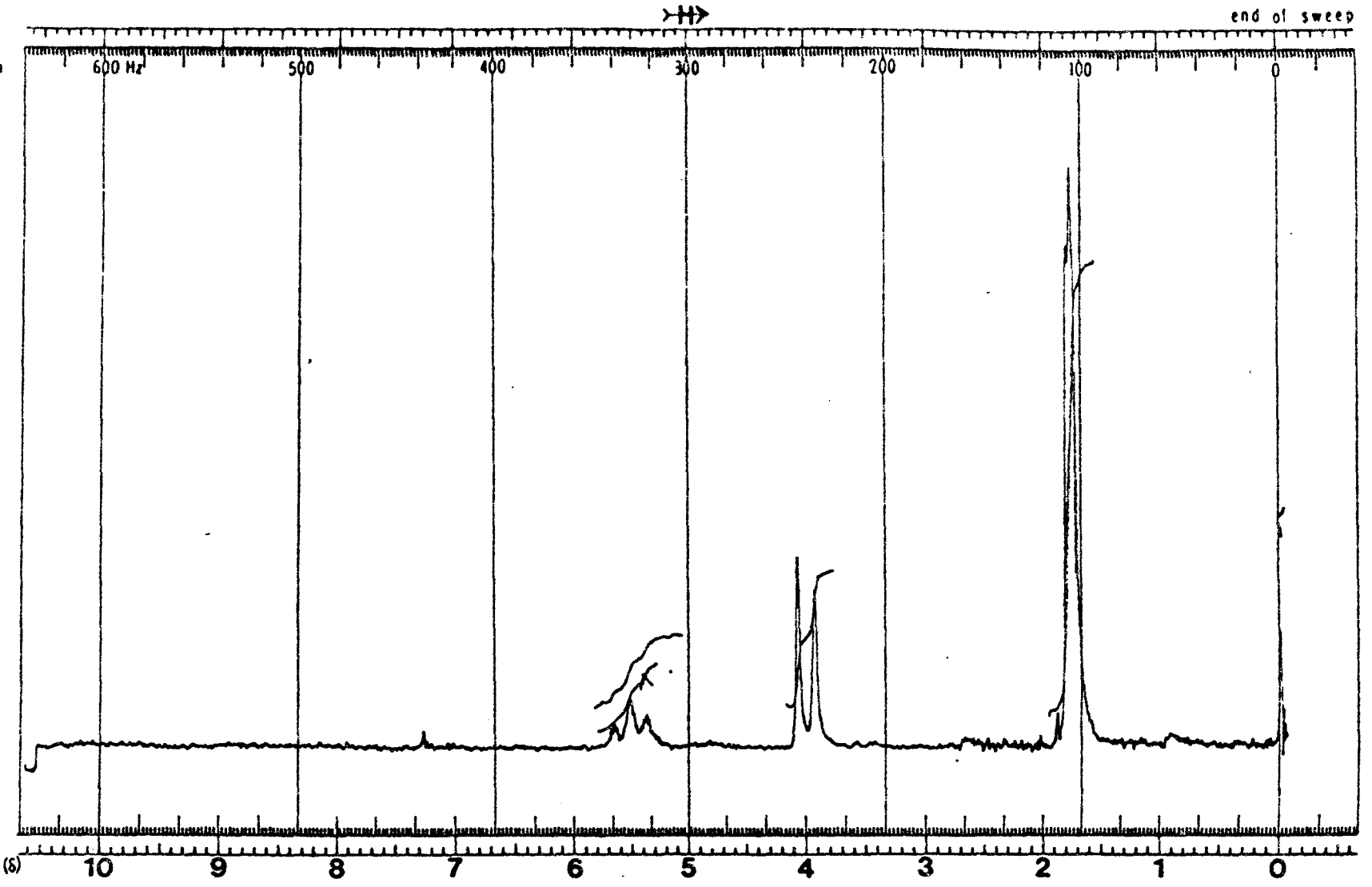
(Davisson et al., 1985; Davisson et al., 1986).

NMR of reagents and DMAPP Since dimethylallyl bromide (1-bromo-3-methyl-2-butene) is the key reagent and the pyrophosphate salt [tris (tetra-n-butylammonium) hydrogen pyrophosphate] is the key intermediate in the synthesis of organic diphosphates, these compounds were identified by ^{1}H NMR prior to their use in the synthesis of DMAPP. The spectrum obtained for dimethylallyl bromide is shown in Figure 6. The spectrum of the pyrophosphate salt (Figure 7) indicated that the sample was tainted with water and contained some impurities. However, the spectrum clearly demonstrated that the pyrophosphate salt was present. No attempts were made to remove the water from the pyrophosphate salt since water of hydration is naturally present in pyrophosphate salts after their synthesis and attempts to remove any additional water results in their decomposition (Davisson et al., 1985; Davisson et al., 1986).

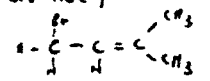
The material recovered after synthesis and lyophilization was checked by ^{31}P NMR. Spectra obtained by ^{31}P NMR showed that the compound indeed contained a pyrophosphate (Figure 8). This was important because the pyrophosphate residues in the DMAPP are superb leaving groups, especially when protonated, and this is one of the reinforcing factors that makes its synthesis difficult

Figure 6: ^{1}H NMR spectrum of dimethylallyl bromide (1-bromo-3-methyl-2-butene). Spectrum was run in $CDCl_3$ at 25°C.

INSTRUMENT: EM-360 _____ EM-360A _____



SPECTRUM AMPL 10x10 SWEEP TIME 50 min SAMPLE: 1-bromo-3-methyl-2-butene (dimethylallyl bromide)



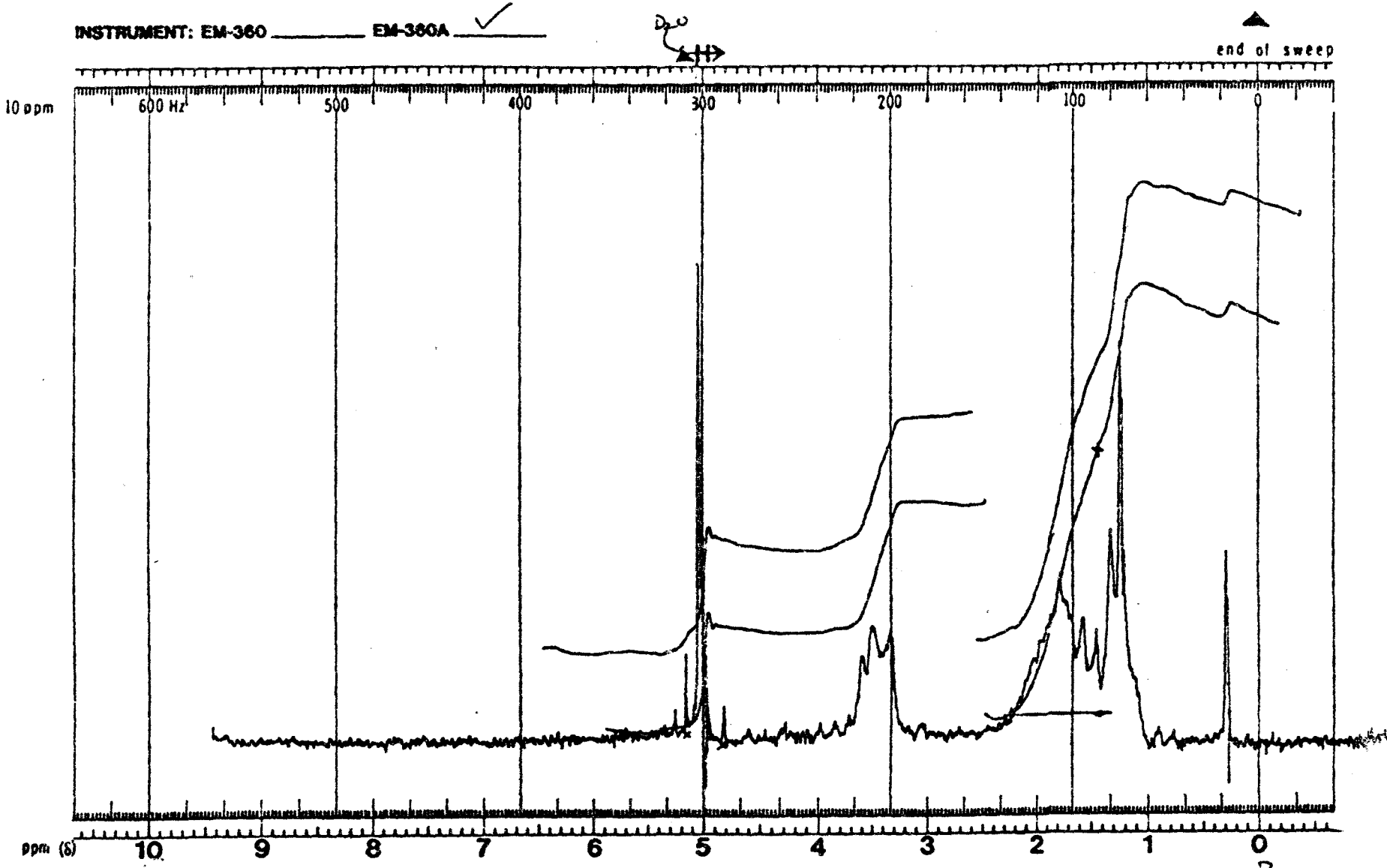
OPERATOR Ray

FILTER 0.1 SWEEP WIDTH 10 ppm or Hz DATE 4/23/88

RF POWER 0.1 END OF SWEEP 0 ppm or Hz SOLVENT: $CDCl_3$

SPECTRUM NO 2

Figure 7: ^{1}H NMR of tris (tetra-n-butylammonium) hydrogen pyrophosphate. Spectrum was run in D_2O adjusted to pH 8.0 with ammonium deuteroxide-d_5 at 25°.

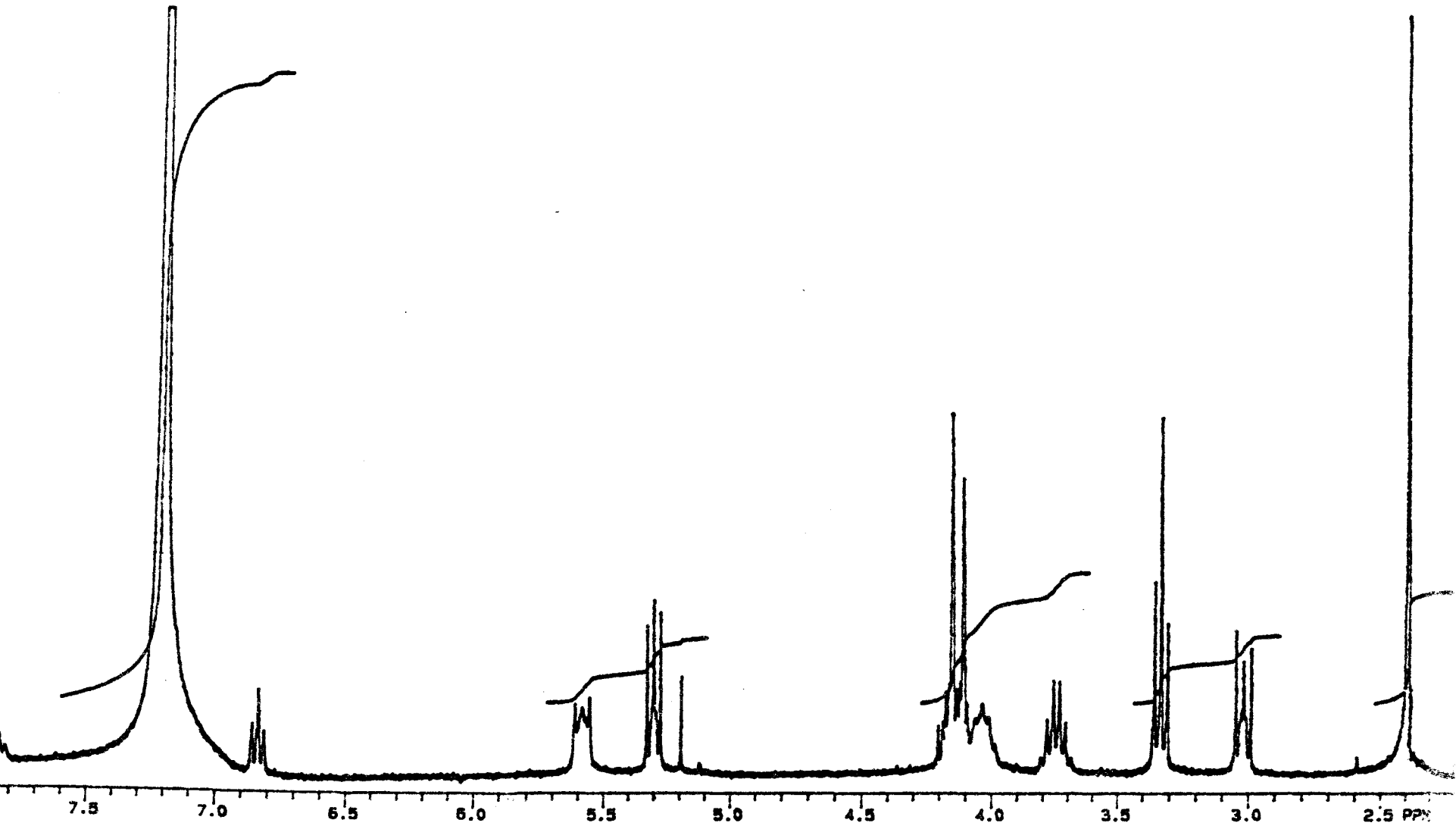


SPECTRUM AMPL 1 1/2 x 2 | SWEEP TIME 5 min | SAMPLE: Tris (tetra-n-butyl) ammonium hydrogen pyrophosphate | OPERATOR Ray

FILTER 0.1 | SWEEP WIDTH 10 ppm or Hz | DATE 21/6/88

RF POWER 0.05 | END OF SWEEP 2 ppm or Hz | SOLVENT: D2O | SPECTRUM NO

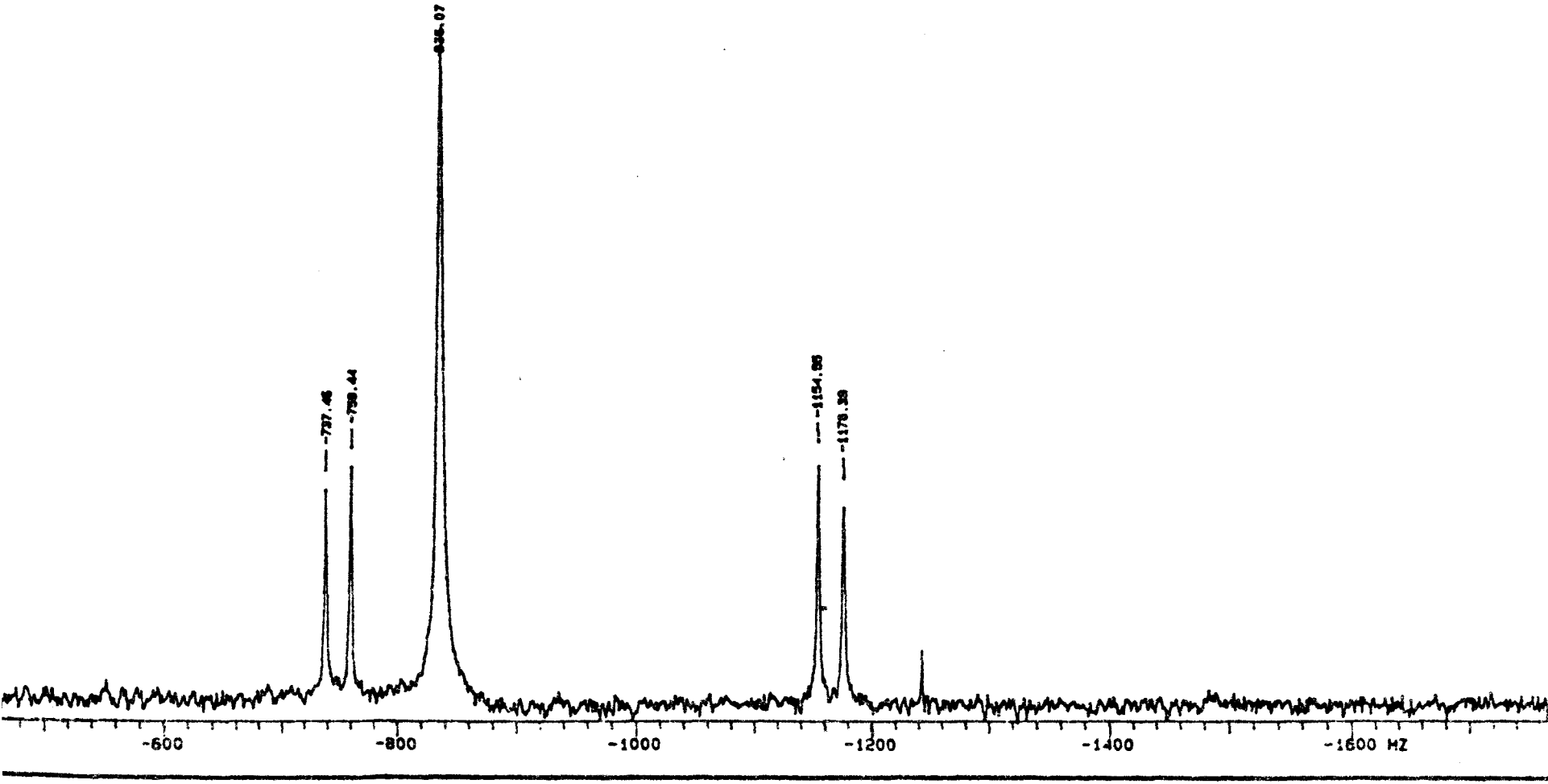
Figure 8: ^{31}P NMR of DMAPP. Spectrum was run in D_2O adjusted to pH 8.0 with ammonium deuteroxide-d_5 at 25°.



1.788	300	1.788	300.3	32	—	—	STDSH	
4000.0	700	NON	20	—	—	—		RAYH
3.752	0	C	200	1735.4	527.7	—		H OF RAYB SAMPLE

(Davisson et al., 1985; Davisson et al., 1986). The ^{31}P NMR spectra, however, showed that the DMAPP contained residual tris (tetra-n-butylammonium) hydrogen pyrophosphate. A second run through a fresh cation-exchange column combined with the organic extraction procedure (which removed any ammonium salts from the isoprenoid diphosphates) removed this impurity. This can be seen in the ^{1}H NMR spectrum (Figure 9). After these steps, the synthesized DMAPP was relatively free of organic and inorganic impurities (Figure 9).

Figure 9: ^{1}H NMR of DMAPP. Spectrum was run in D_2O adjusted to pH 8.0 with ammonium deuteroxide-d_5 at 25°.



PP or P_i ?

R-O-P-O-P

DMAPP:5'-AMP Transferase Assay

All DMAPP:5'-AMP transferase assays consisted of measuring the amount of isopentenyladenosine-5'-monophosphate (or isopentenyladenosine) produced by incubating the transferase enzyme with DMAPP and radiolabelled 5'-AMP. A diagram of this reaction can be seen in Figure 10. Since free cytokinins in S. cerevisiae are present at exceedingly low levels (Laten and Zahareas-Doktor, 1985), it would be expected that the enzymes that synthesize them are present at low levels (Hommes et al., 1985). Furthermore, cytokinin biosynthesis represents a minor pathway of metabolism of the ubiquitous purine adenine, and isolating and purifying the minute quantities of cytokinins from the pool of common purine metabolites and degradation products requires both an assay with high specificity and stringent purification procedures.

DMAPP:5'-AMP transferase assay 1 - Since the DMAPP:5'-AMP transferase enzyme had never been identified in yeast, the assay parameters of Hommes et al. were employed with minor modifications (Hommes et al., 1985). Twenty grams of frozen S. cerevisiae strain 1480 yeast paste was broken by toluene autolysis (referred to as crude extract), and subjected to ammonium sulfate fractionation. The precipitate obtained between 25-80% saturation was then extensively dialyzed against buffer #1 (see Materials and Methods). No ion-exchange column was used to purify the

Figure 10: DMAPP:5'-AMP transferase assay.

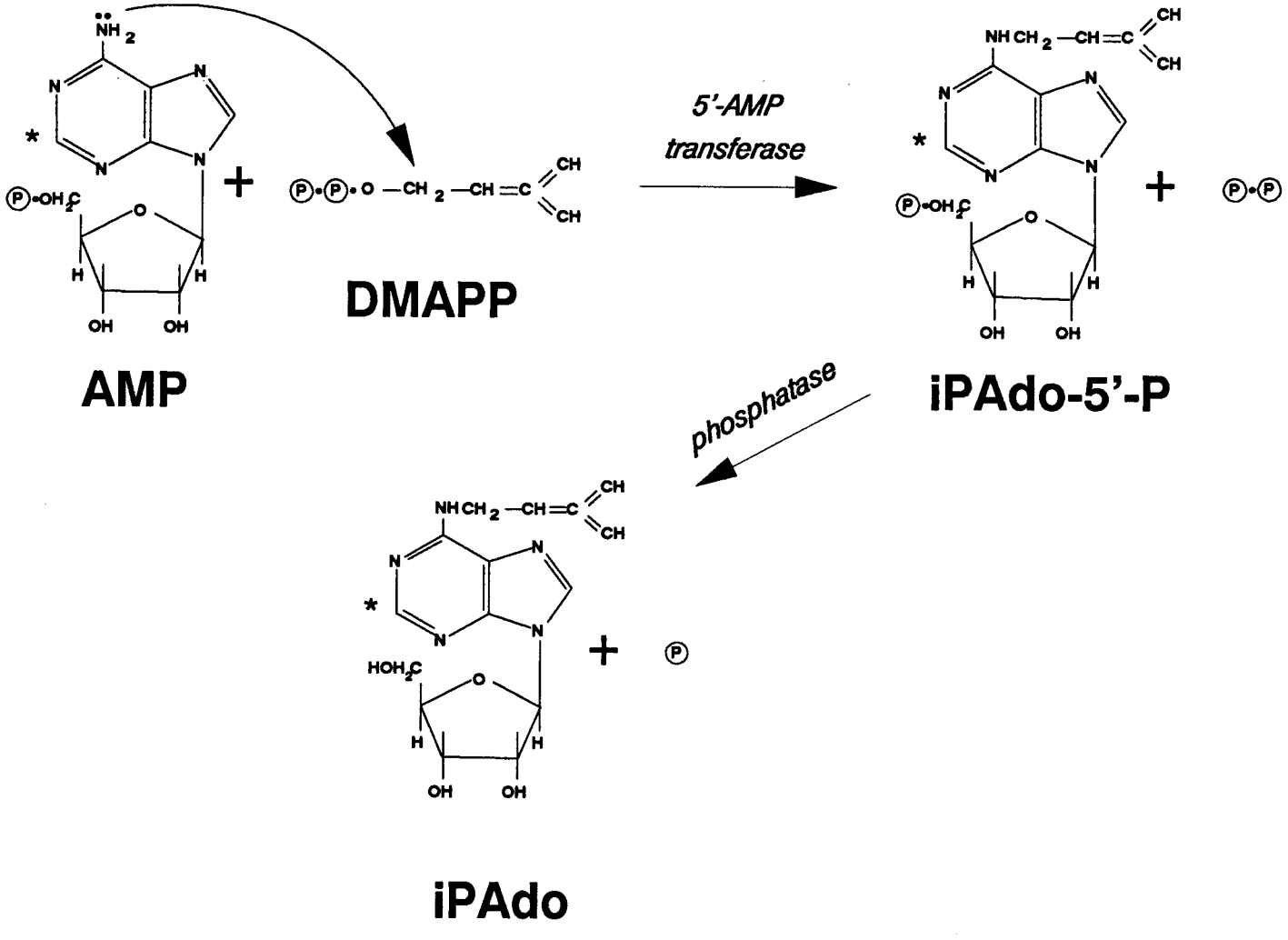


Figure 10.

crude extract further. This nucleic acid free protein preparation (now referred to as crude homogenate) contained 15 μg/ul protein and was assayed for DMAPP:5'-AMP transferase products. The assay mixture was incubated for 28 hours at 30°C, dephosphorylated, and subjected to analysis by HPLC. Results from the DMAPP:5'-AMP transferase assay 1 are shown in Figure 11 and Figure 12.

The results from figure 11 and 12 show that there is no iPAMP peak in the assay with DMAPP. This assay was repeated (DMAPP:5'-AMP Transferase Assay 2) in an attempt to obtain better results.

Figure 11: Assay 1 elution profile of Saccharomyces cerevisiae mod5 strain 1480 cell-free extracts on HPLC. Samples were run on a reverse phase ODS C_{18} column using the following gradient program (flow rate, 1 ml/min): 5%-20% methanol over 20 min., 20%-80% over 20 min. The sample was then dissolved in 100 ul 95% methanol and applied to the column. Retention times of cytokinin standards are indicated. Abbreviations: iPAMP, isopentenyladenosine-5'monophosphate; iPAdo, isopentenyladenosine; iPAde, isopentenyladenine.

Figure 12: Assay conditions identical to Figure 11 except reaction contains no DMAPP. Elution conditions identical to Figure 11.

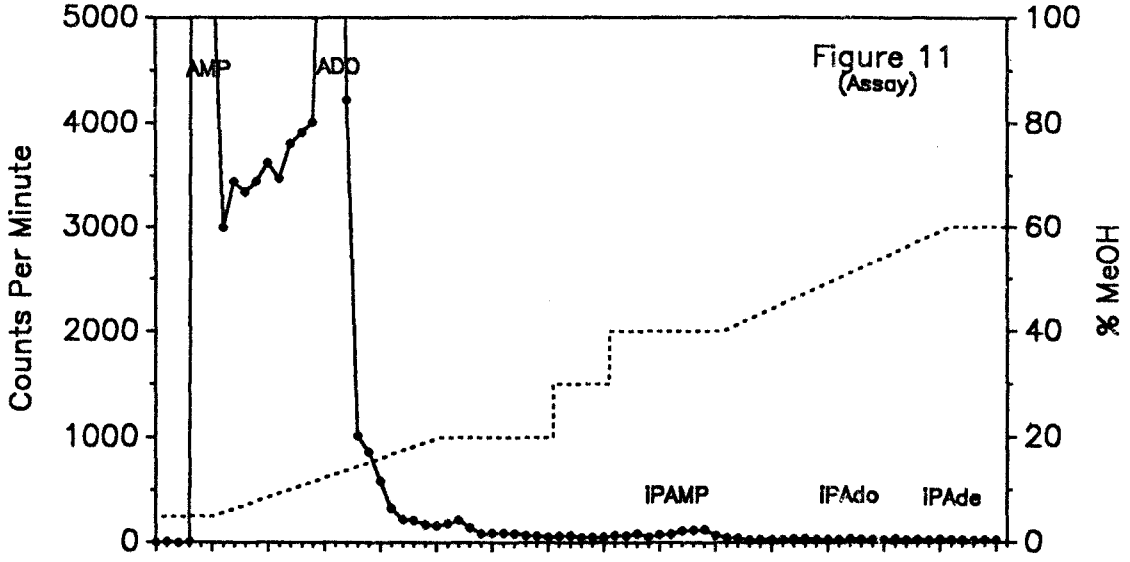


Figure 11
(Assay)

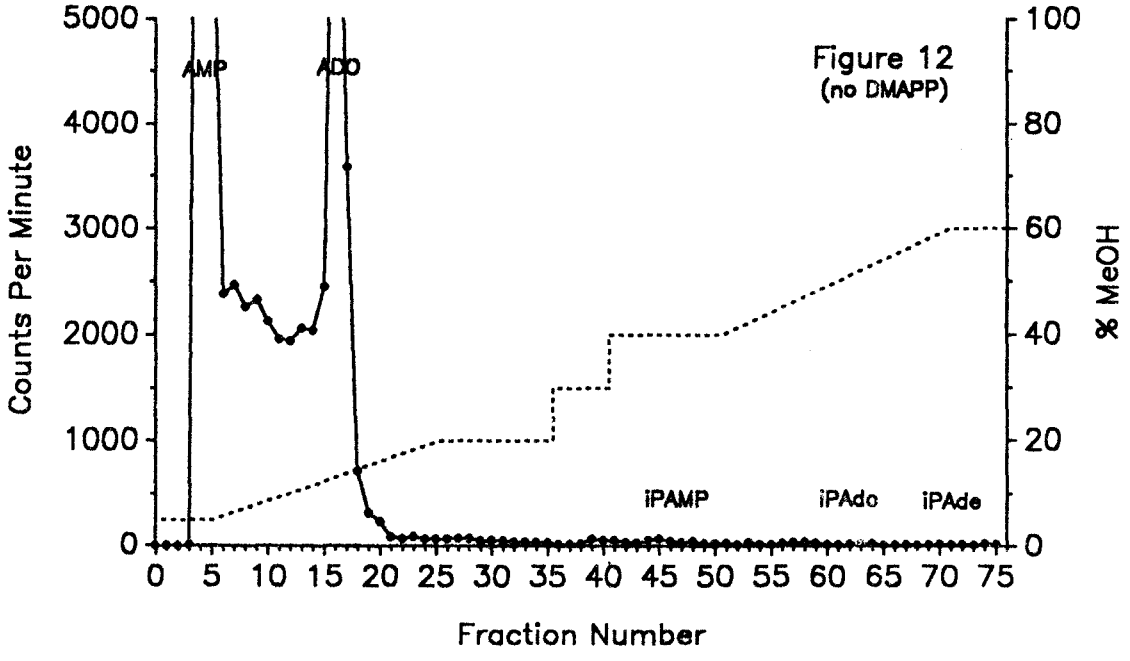


Figure 12
(no DMAPP)

DMAPP:5'-AMP transferase assay 2 - This assay was identical to assay 1 with the exception that the crude homogenate contained 60.5 mg of protein. Results from the DMAPP:5'-AMP transferase assay 2 are shown in Figure 13 and Figure 14.

These results show that there is no significant difference between the assay and control runs. There was no significant difference in the amount of label that comigrated with iPAdo or iPAMP in the assays whether or not DMAPP was present. A continuous culture of S. cerevisiae strain 1480 was then grown in an attempt to study the transferase enzyme in newly harvested yeast.

DMAPP:5'-AMP transferase assay 3 - This assay was identical to assay 2 with the exception that the crude homogenate contained 81.25 mg of protein isolated from yeast within 48 hours of the assay. Results for DMAPP:5'-AMP transferase assay 3 are shown in Figure 15 and Figure 16.

Assay 3 failed to show any detectable DMAPP:5'-AMP transferase products even though newly harvested yeast was used.

Figure 13: Assay 2 elution profile of Saccharomyces cerevisiae mod5 strain 1480 cell-free extracts on HPLC. Abbreviations: AMP, adenosine-5'-monophosphate; Ado, adenosine.

Figure 14: Assay conditions identical to Figure 13 except reaction contains no DMAPP. Elution conditions identical to Figure 13.

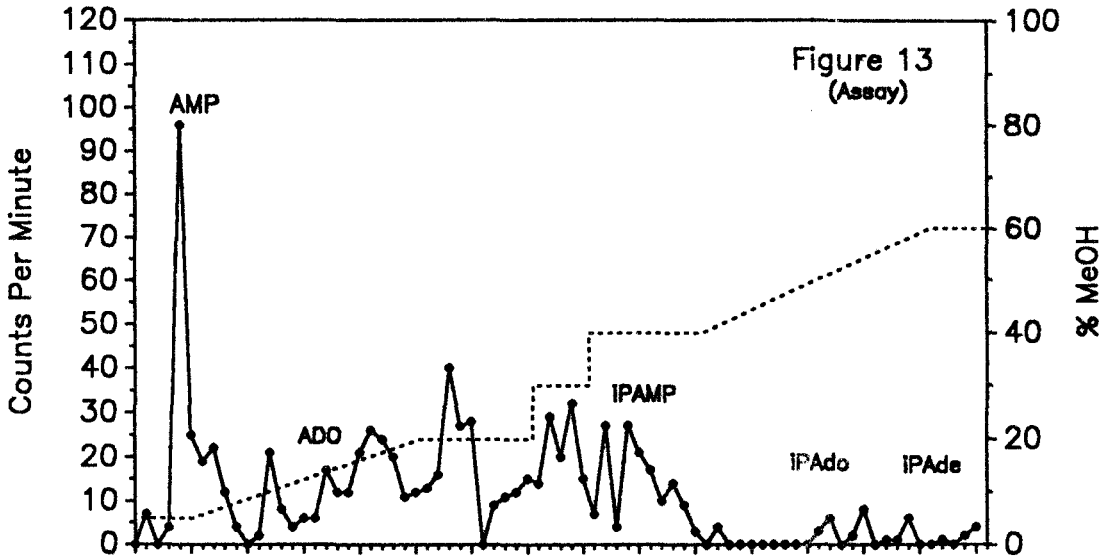


Figure 13
(Assay)
AMP
ADO
IPAMP
IPAdo
IPAde
Counts Per Minute
% MeOH

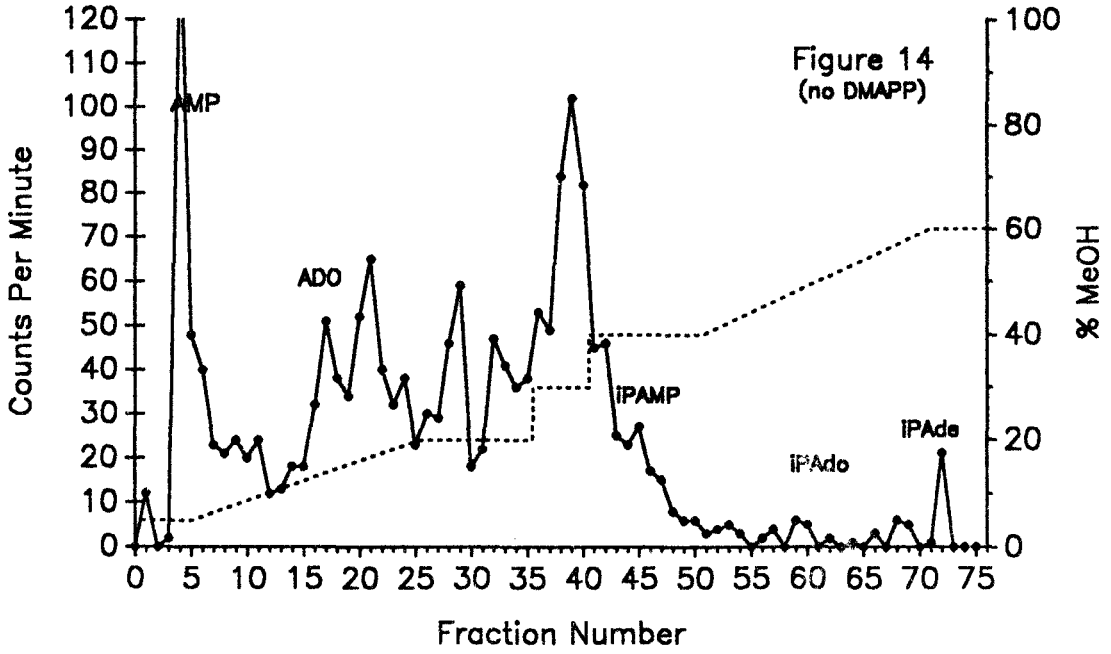


Figure 14
(no DMAPP)
AMP
ADO
IPAMP
IPAdo
IPAde
Counts Per Minute
% MeOH
Fraction Number

Figure 15: Assay 3 elution profile of Saccharomyces cerevisiae mod5 strain 1480 cell-free extracts on HPLC. Retention times of authentic cytokinins are indicated. Missing fractions 26-32 due to faulty fraction collector.

Figure 16: Assay conditions identical to Figure 15 except reactions contain no DMAPP. Elution conditions identical to Figure 15.

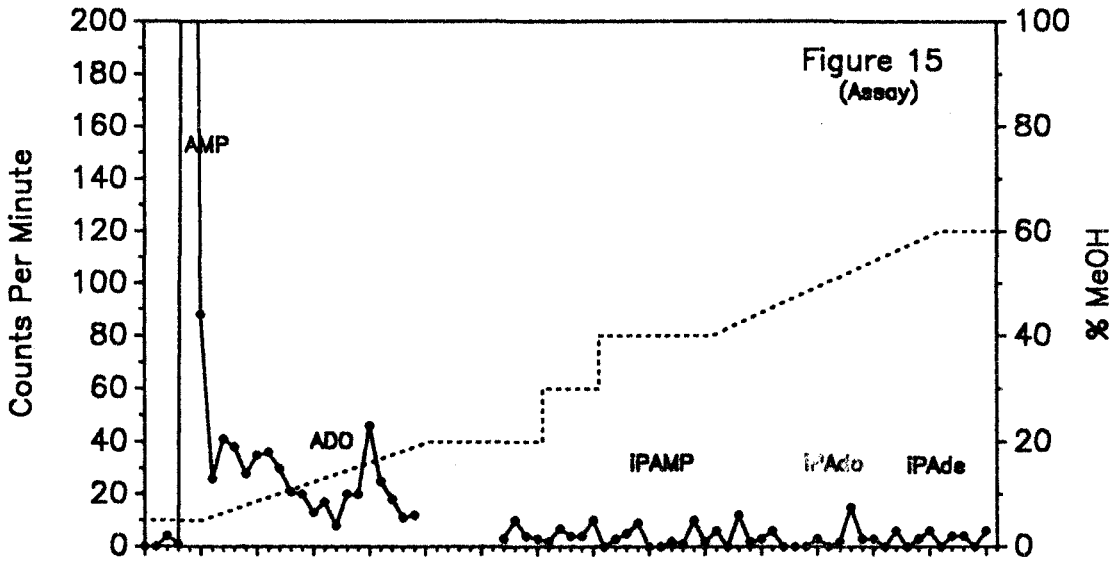


Figure 15
(Assay)
Counts Per Minute
% MeOH
AMP
ADO
IPAMP
iPAdo
iPAde
200
180
160
140
120
100
80
60
40
20
0
100
80
60
40
20
0

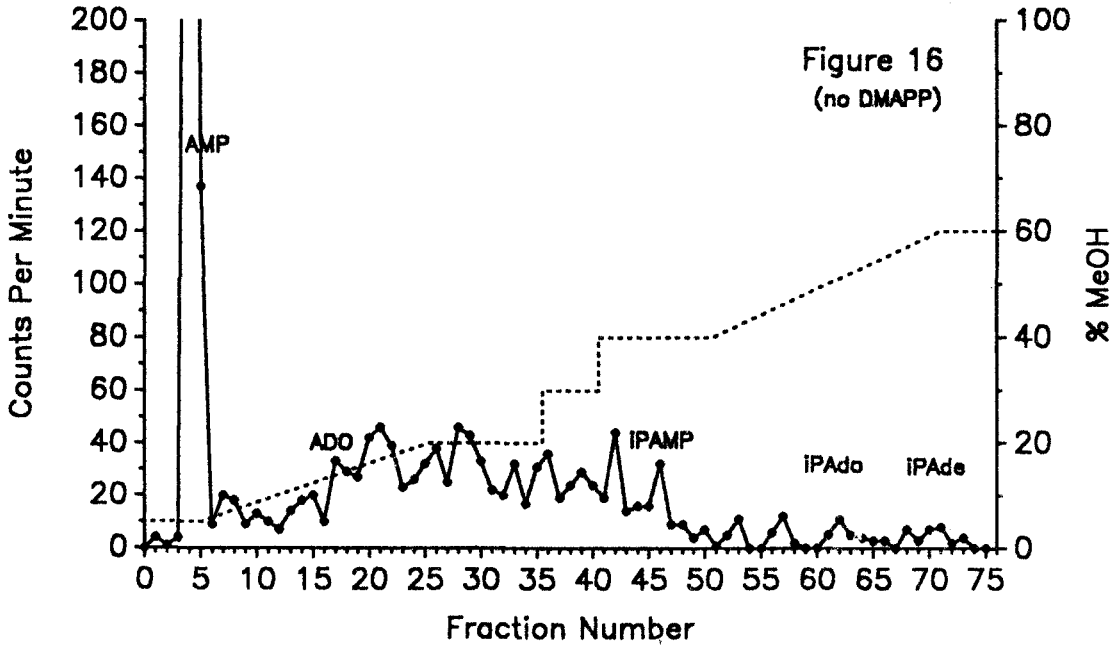


Figure 16
(no DMAPP)
Counts Per Minute
% MeOH
AMP
ADO
IPAMP
iPAdo
iPAde
200
180
160
140
120
100
80
60
40
20
0
100
80
60
40
20
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75
Fraction Number

DMAPP:5'-AMP transferase assay 4 - An effort was made to increase the protein concentration of the crude homogenate prior to assaying for DMAPP:5'-AMP transferase products. After ultrafiltration of the crude homogenate (see Materials and Methods) the protein concentration increased from 12.1 to 54 mg/ml. The concentrated crude homogenate was then assayed for DMAPP:5'-AMP transferase products within 48 hours of isolation from actively growing cells. Nonradioactive carrier (5'-AMP) was omitted from the assay mixture, DMAPP concentration was lowered from 0.6 to 0.15 mM, 5'-[2-^{3}H]AMP concentration was doubled, and the reaction volume was halved from 5.0 ml to 2.5 ml. The assay mixture was incubated for 8 hours at 30°C, dephosphorylated with 20 units BAP, and subjected to analysis by HPLC. Results from the DMAPP:5'-AMP transferase assay 4 are shown in Figure 17 and Figure 18. Results shown in Figures 17 and 18 indicate no detectable DMAPP:5'-AMP transferase products in assay 4. Again, there was not a significant difference in the amount of labelled iPAdo or iPAMP whether or not DMAPP was present. Surprisingly, very little of the unreacted AMP was converted to adenosine, as the vast majority of the counts eluted as AMP.

Figure 17: Assay 4 elution profile of Saccharomyces cerevisiae mod5 strain 1480 cell-free extracts on HPLC. Retention times of cytokinin standards are indicated.

Figure 18: Assay conditions identical to Figure 17 except reaction contains no DMAPP. Elution conditions identical to Figure 17.

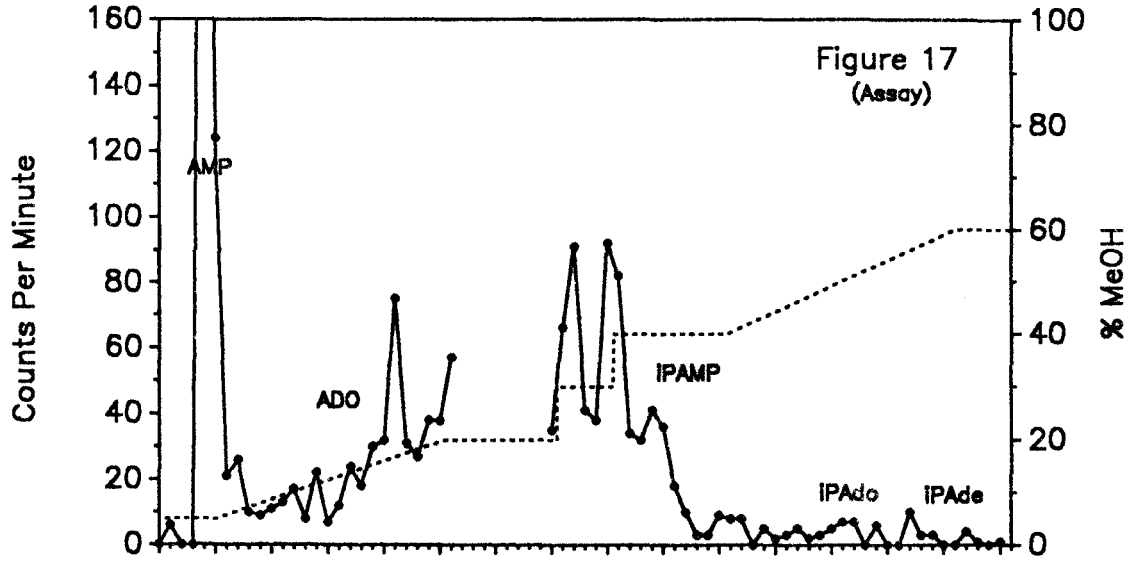


Figure 17
(Assay)
Counts Per Minute
% MeOH
AMP
ADO
IPAMP
IPAdo
IPAde
160
140
120
100
80
60
40
20
0
100
80
60
40
20
0

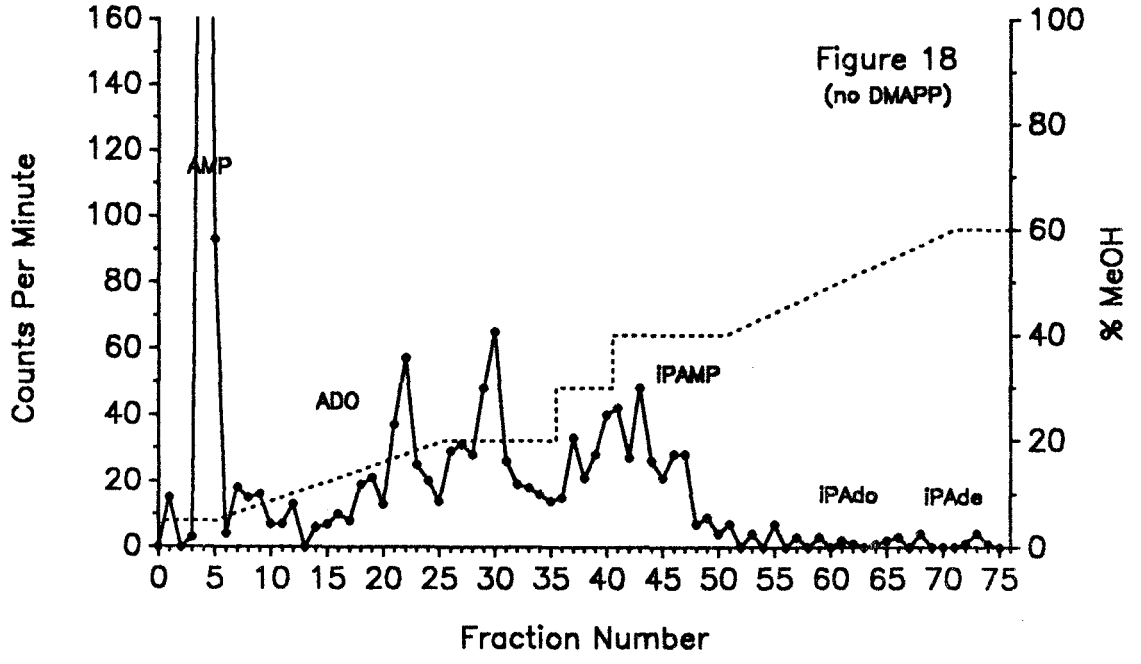


Figure 18
(no DMAPP)
Counts Per Minute
% MeOH
AMP
ADO
IPAMP
IPAdo
IPAde
160
140
120
100
80
60
40
20
0
100
80
60
40
20
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75
Fraction Number

Only a small fraction of the counts were metabolized to reaction products, most of which were unknown. The BAP did not appear to be converting the AMP to adenosine.

DMAPP:5'-AMP transferase assay 5 - Assays were then performed to alter the 5'-AMP concentration to adjust the BAP to proper K_m range. One mM 5'-AMP was found to work best, which coincided with the K_m for BAP using 5'-AMP as a substrate reported by Heppel and Harkness (1961). Proper concentration of DMAPP was also tested by varying the amounts of DMAPP added. The assay mixture contained 135 mg of protein, and the assay was incubated for 8 hours. The reaction mixture was then subjected to analysis by HPLC. Results from the DMAPP:5'-AMP transferase assay 5 are shown in Figure 19 and Figure 20. The addition of the 1 mM 5'-AMP had a marked effect on the breakdown of phosphate groups. A greater portion of the 5'-AMP was turned over into other metabolites, most noticeably adenosine. Unfortunately, there were no detectable DMAPP:5'-AMP transferase products, as no labelled cytokinins could be detected.

Figure 19: Assay 5 elution profile of Saccharomyces cerevisiae mod5 strain 1480 cell-free extracts on HPLC. Retention times of cytokinin standards are indicated by the boxes.

Figure 20: Assay conditions identical to Figure 19 except reaction contains no DMAPP. Elution conditions identical to Figure 19.

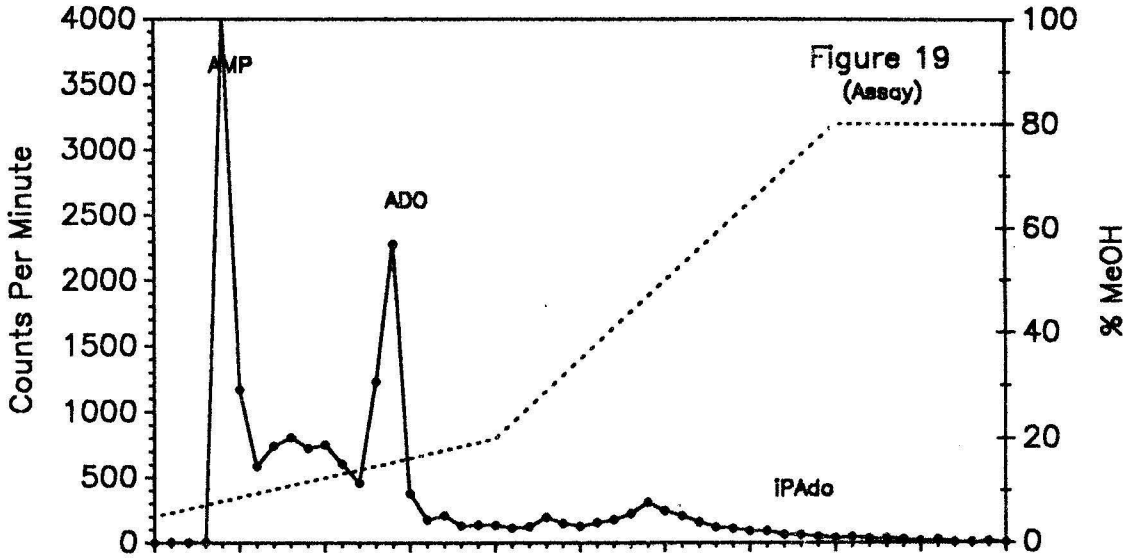


Figure 19
(Assay)
AMP
ADO
iPAdo
Counts Per Minute
% MeOH
4000
3500
3000
2500
2000
1500
1000
500
0
100
80
60
40
20
0

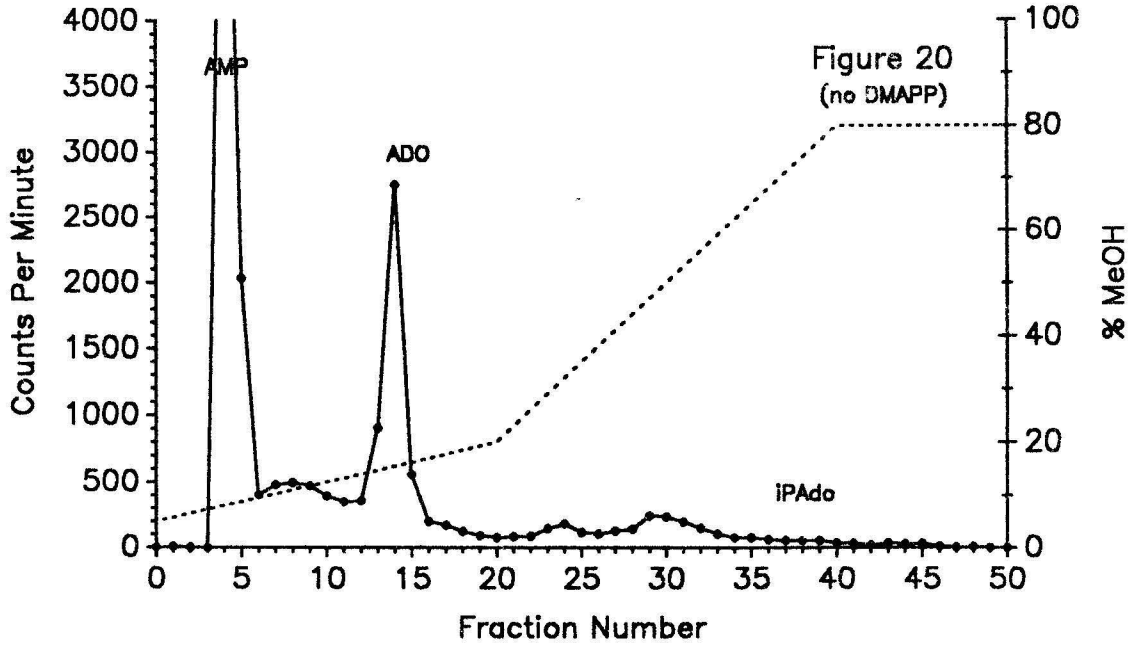


Figure 20
(no DMAPP)
AMP
ADO
iPAdo
Counts Per Minute
% MeOH
Fraction Number
4000
3500
3000
2500
2000
1500
1000
500
0
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
45
50

Enzyme assays performed up to this point failed to show any DMAPP:5'-AMP transferase products. A means to test assay parameters was needed at this point to show if a previously characterized DMAPP:5'-AMP transferase product could be detected under current conditions employing our reagents. E. coli strain HB101 (pTHH21) was utilized carrying a cosmid clone of pTiC18, which contains the ipt region. It served as a positive control.

DMAPP:5'-AMP transferase assay 6 -Twenty grams of frozen HB101 (pTHH21) was broken by bead disruption, and an assay and partial purification of the DMAPP:5'-AMP transferase was then performed according to the parameters in DMAPP:5'-AMP transferase assay 1 [Hommes, et al. (1985)]. Analysis of the reaction products was then performed by thin layer chromatography. Results from the DMAPP:5'-AMP transferase assay 6 employing HB101 (pTHH21) are shown in Figure 21.

Figure 21: Reaction products from DMAPP:5'-AMP transferase assay 6 employing cosmid clone pTiC18, which overlaps the ipt region. Lane 2, DMAPP:5'-AMP transferase assay; lane 3, control (no DMAPP) The autoradiofluorogram was exposed 5 days at -78°C.

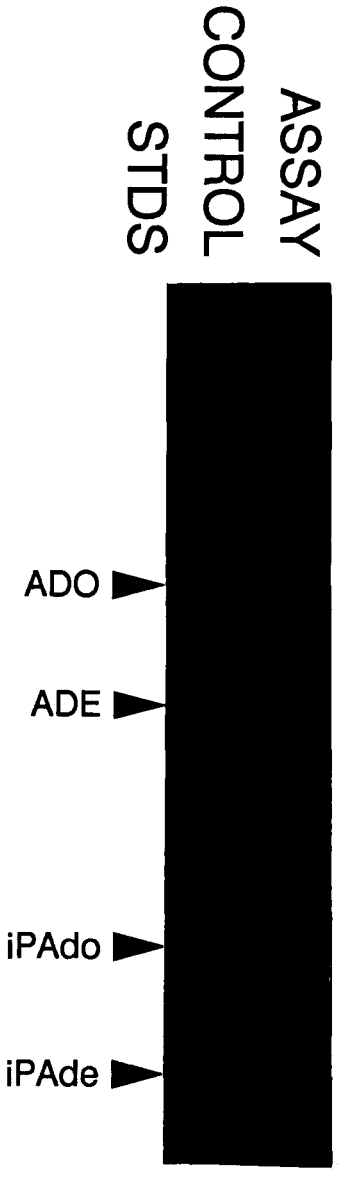


Figure 21.

Addition of DMAPP and 5'-[2-^{3}H]AMP to cell-free extracts of E. coli containing a cloned copy of the ipt gene results in the incorporation of radioactivity into cytokinins. The lower band on the autofluorogram coelutes with authentic iPAdo. DMAPP:5'-AMP transferase products are indeed shown in this extract.

Since the S. cerevisiae crude homogenate was not showing any transferase products, an effort was made to purify the transferase enzyme further in hopes of removing any inhibitors that may be masking transferase products.

DMAPP:5'-AMP transferase assay 7 -DEAE cellulose ion exchange chromatography was employed to further purify crude homogenate and eliminate the bulk of the impurities (Morris, et al., 1982; Akiyoshi, et al., 1983) from S. cerevisiae strain 1480 and E. coli HB101 (pTHH21). Assays were performed according to the parameters in DMAPP:5'-AMP transferase assay 1 [Hommes, et al. (1985)]. Visualization of reaction products was performed on thin layer chromatography plates. Results can be seen in Figure 22.

Results from assay 7 show that DMAPP:5'-AMP transferase products are detected in HB101-pTHH21 fractions obtained before and after the DEAE purification step, but not detectable in the S. cerevisiae assays either before or after the DEAE column. Addition or subtraction of DMAPP had no effect in the yeast transferase assays.

Figure 22: Reaction products from DMAPP:5'-AMP transferase assay 7 employing S. cerevisiae strain 1480 and E. coli HB101 (pTHH21). Lane 1, 1480 control (no DMAPP) after DEAE; lane 2, 1480 assay after DEAE; lane 3, 1480 control (no DMAPP) before DEAE; lane 4, 1480 assay before DEAE; lane 5, HB101 (pTHH21) control (no DMAPP) after DEAE; lane 6, HB101 (pTHH21) assay after DEAE; lane 7, HB101 (pTHH21) control (no DMAPP) before DEAE; lane 8, HB101 (pTHH21) assay before DEAE; lane 9, standards: ade, ado, iPAde, iPAdo.

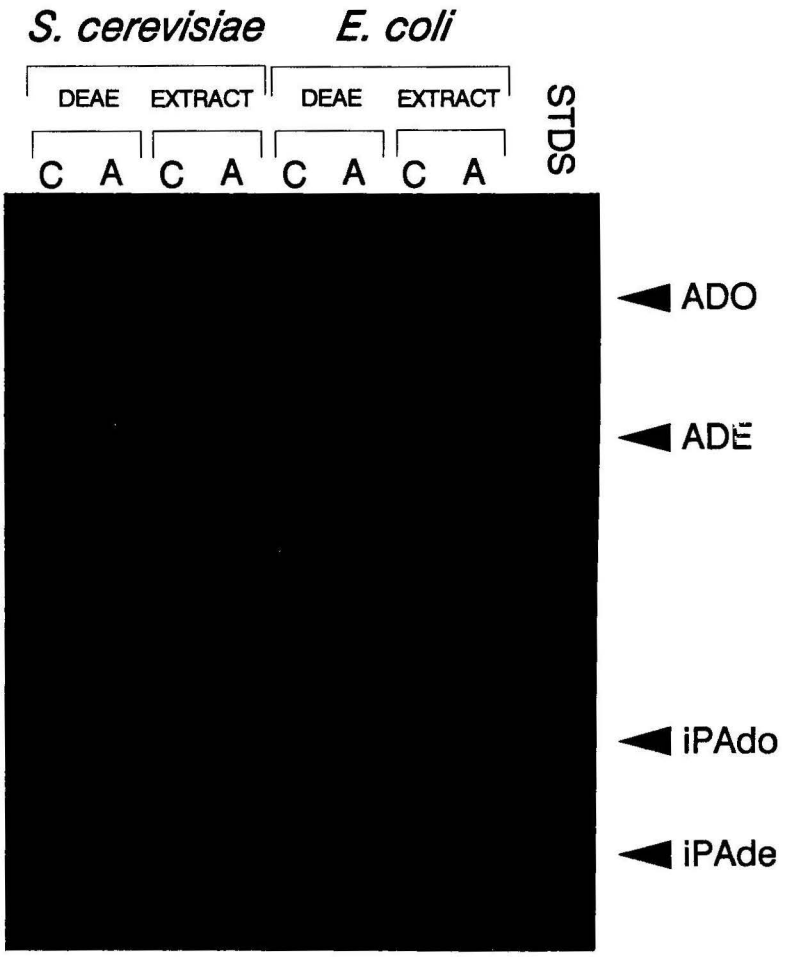

S. cerevisiae
E. coli
DEAE
EXTRACT
DEAE
EXTRACT
C A C A C A C A
STDS
ADO
ADE
iPAdo
iPAde

Figure 22.

DMAPP:5'-AMP transferase assay 8 - Level of detection and lability of DMAPP:5'-AMP transferase was tested. Six week and three week old crude homogenate prepared from recombinant E. coli and purified through a DEAE column was tested for transferase products according to the parameters of Hommes et al., 1985. Visualization of reaction products was again performed on thin layer chromatography plates. Results can be seen in Figure 23. Addition of DMAPP and 5'-[2-^{3}H]AMP to three week old cell-free extracts of E. coli HB101 (pTHH21) results in the incorporation of radioactivity into cytokinins (lane 7 and 9). Six week old crude homogenate showed no detectable transferase products (lanes 2-5). Both 1 and 2 ml fractions of three week old crude homogenate showed transferase enzyme products (lanes 7 and 9, respectively). Hence crude homogenate is very unstable and exhibits no transferase products between 3 and 6 weeks after DEAE cellulose chromatography. Furthermore, as little as 1 ml crude homogenate (1.8 mg protein/ml) is necessary to detect transferase products.

Figure 23: Reaction products from DMAPP:5'-AMP transferase assay 8 employing E. coli HB101 (pTHH21). Lane 1, standards: ade, ado, iPAde, iPAdo; lane 2, 1.0 ml 6 week HB101 (pTHH21) control (no DMAPP); lane 3, 1.0 ml 6 week HB101 (pTHH21) assay; lane 4, 2.0 ml six week HB101 (pTHH21) control (no DMAPP); lane 5, 2.0 ml six week HB101 (pTHH21) assay; lane 6, 1.0 ml three week HB101 (pTHH21) control; lane 7, 1.0 ml three week HB101 (pTHH21) assay; lane 8, 2.0 ml three week HB101 (pTHH21) control (no DMAPP); lane 9, 2.0 ml three week HB101 (pTHH21) assay.

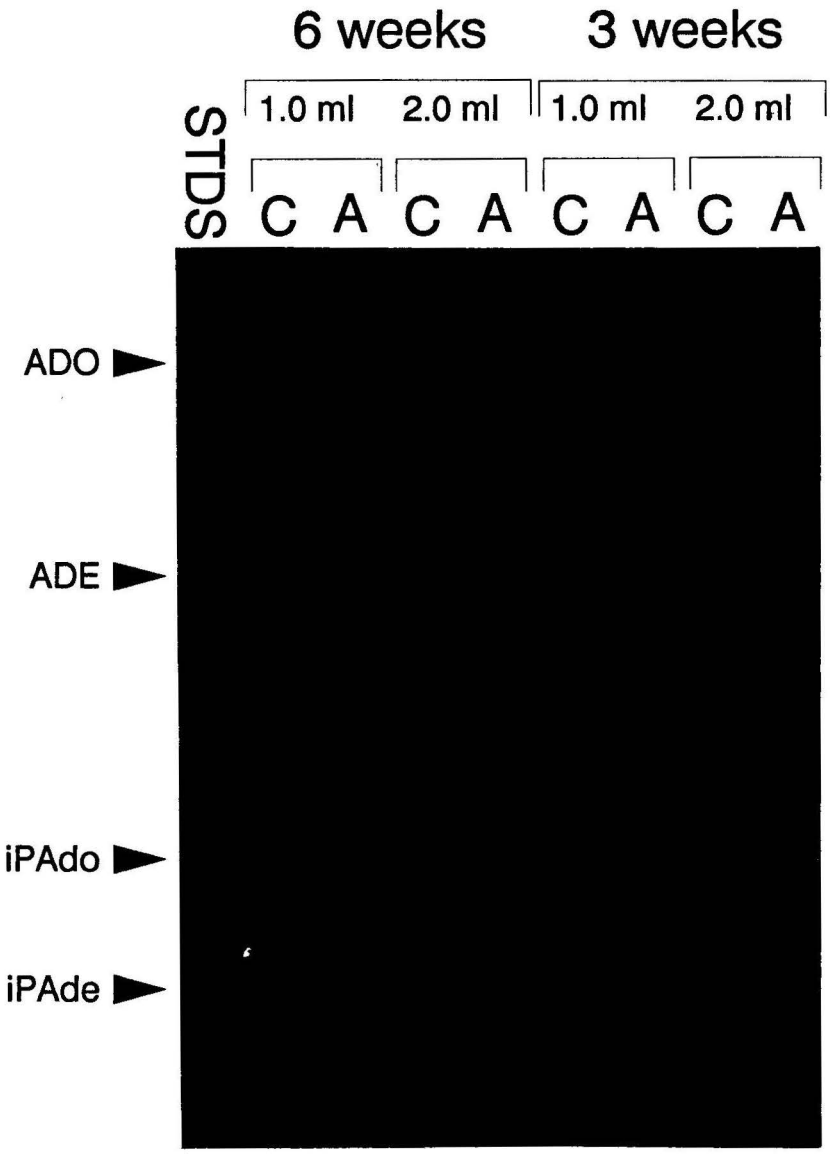

6 weeks
3 weeks
1.0 ml
2.0 ml
1.0 ml
2.0 ml
STDS
C A C A C A C A
ADO
ADE
iPAdo
iPAde

Figure 23.

Purification by DEAE cellulose ion exchange chromatography produced fractions with active enzyme products. An assay was developed in order to verify where the active fractions eluted from the column.

DMAPP:5'-AMP transferase assay 9 - A DEAE column was loaded with crude homogenate from either S. cerevisiae strain 1480 or E. coli HB101 (pTHH21) and eluted with 0.3 M KCl. Fractions from the first and second half were collected and pooled. An enzyme assay for DMAPP:5'-AMP transferase was then performed. Results from this assay are shown in Figure 24.

Addition of DMAPP and 5'-[2-^{3}H]AMP to the first half DEAE eluent fraction of E. coli HB101 (pTHH21) resulted in the incorporation of radioactivity into both iPAdo and iPAde (lane 6). Interestingly, the second half DEAE eluent fraction is only able to incorporate radioactivity into iPAdo (lane 8). The first fractions that eluted from the column seem to be the more active fractions although both reveal DMAPP:5'-AMP transferase products.

Figure 24: Pooled reaction products from DMAPP:5'-AMP transferase assay 9 employing E. coli HB101 (pTHH21) (lanes 1-4) and S. cerevisiae strain 1480 (lanes 5-8). Lane 1, HB101 (pTHH21) second half control. lane 2, HB101 (pTHH21) second half assay; lane 3, HB101 (pTHH21) first half control; lane 4, HB101 (pTHH21) first half assay; lane 5, 1480 second half control; lane 6, 1480 second half assay; lane 7, 1480 first half control; lane 8, 1480 first half assay; lane 9, standards: ade, ado, iPAde, iPAdo.

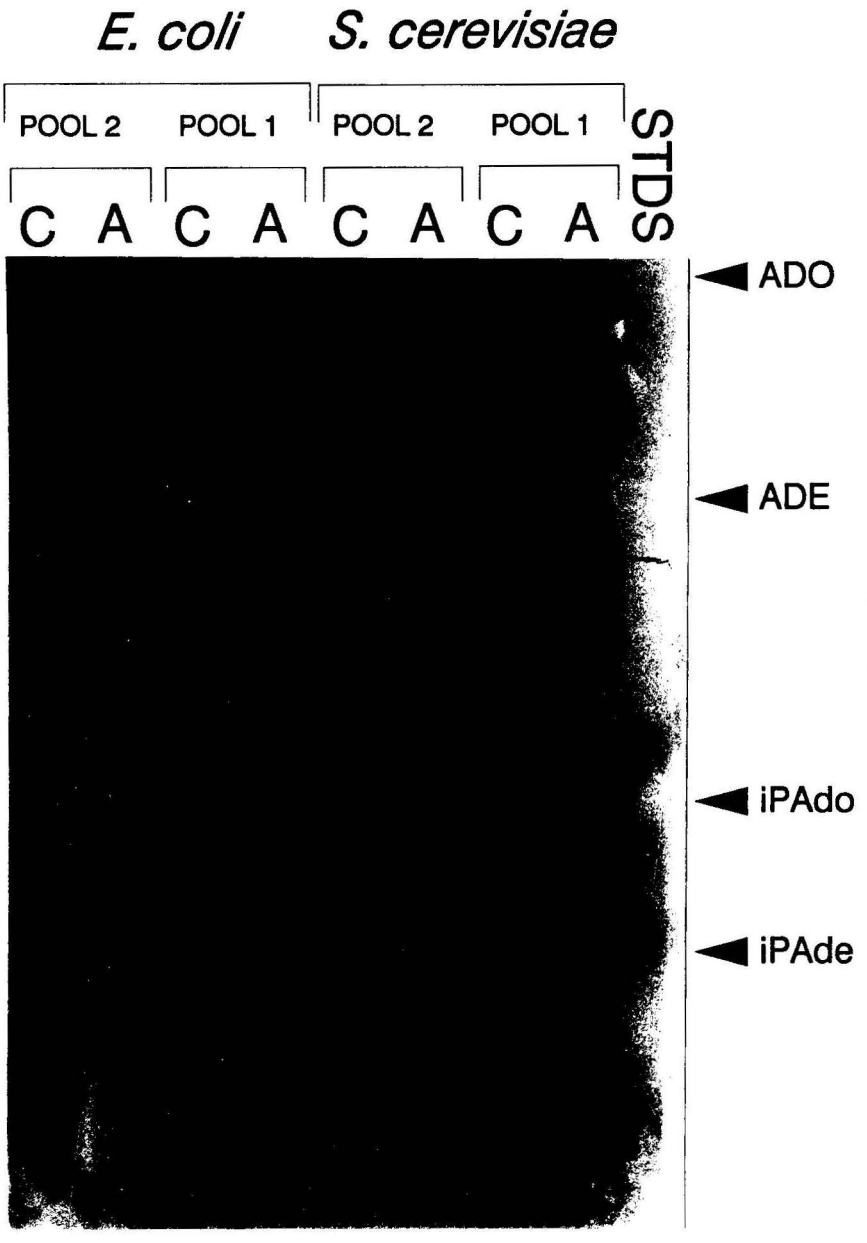

E. coli
S. cerevisiae
POOL 2
POOL 1
POOL 2
POOL 1
STDS
C A C A C A C A
ADO
ADE
iPAdo
iPAde

Figure 24.

The assays containing S. cerevisiae 1480 fractions showed no DMAPP:5'-AMP transferase products for either the first half fractions (lane 2) or the second half fractions (lane 4). The yeast strain had a 10x greater concentration of protein (3.63 mg/ml) than HB101 (pTHH21) (0.300 mg/ml).

To test whether S. cerevisiae may be producing an inhibitor(s) that suppresses DMAPP:5'-AMP transferase products or its detection, I mixed extract from HB101 (pTHH21) with extract from S. cerevisiae strain 1480. If transferase (from HB101 (pTHH21) is not detected in a mixture of both HB101 (pTHH21) and 1480, then a substance in the yeast crude homogenate is inhibiting transferase products or their detection. Both toluene autolysis and bead disruption (bead beater-bb) were used to break open S. cerevisiae cells.

DMAPP:5'-AMP transferase assay 10 - One hundred ninety g of S. cerevisiae were broken by toluene autolysis or bead disruption, mixed with extract from HB101 (pTHH21), and an assay and partial purification of the DMAPP:5'-AMP transferase was then performed according to the parameters in DMAPP:5'-AMP transferase assay 1 [Hommes, et al. (1985)]. Results from this assay are shown in Figure 25. All assays containing HB101 (pTHH21) showed DMAPP:5'-AMP transferase products. The assay run with a mixture of 1480 bead disrupted cell pellets + HB101 (pTHH21) (lane 4) and the

assay run with a mixture of 1480 toluene lysed cell pellets + HB101 (pTHH21) (lane 8) both showed enzyme products, but at lower levels than when HB101 (pTHH21) was assayed alone. Strain 1480 alone, weather toluene lysed or bead disrupted, failed to show any transferase products.

Figure 25: Transferase assay 10 employing mixed reaction products from S. cerevisiae strain 1480 and E. coli HB101 (pTHH21). Lane 1, standards: ado, ade, iPAdo, iPAde; lane 2, 1480 bb assay; lane 3, 1480 bb control; lane 4, 1480 bb+ HB101 (pTHH21) assay; lane 5, 1480 bb+ HB101 (pTHH21) control; lane 6, 1480 toluene assay; lane 7, 1480 toluene control; lane 8, 1480 toluene+ HB101 (pTHH21) assay; lane 9, 1480 toluene+ HB101 (pTHH21) control; lane 10, HB101 (pTHH21) assay; lane 11, HB101 (pTHH21) control.

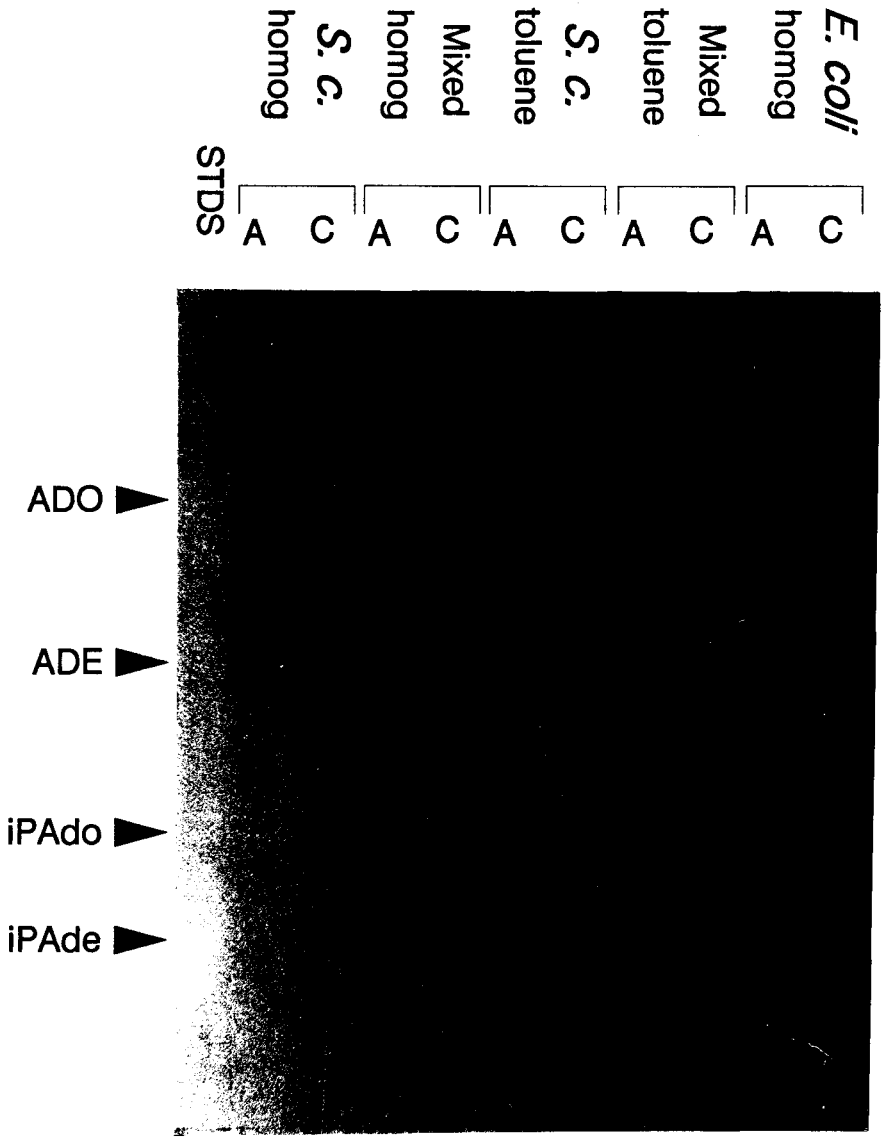

STDS
A C A C A C A C A C
E. coli homog
Mixed toluene
S. c. toluene
Mixed homog
S. c. homog
ADO
ADE
iPAdo
iPAde

Figure 25.

CHAPTER V

DISCUSSION

DMAPP:5'-AMP transferase in Saccharomyces cerevisiae is below the level of detection using standard assay techniques. All attempts to detect transferase products in S. cerevisiae were unsuccessful.

In Transferase assays 1 and 2, no transferase detection may have been due to the fact that the frozen yeast used for this assay run was 6 months old and DMAPP:5'-AMP transferase is exceedingly labile (Chen, 1982; Ihara et al., 1984; Hommes et al., 1985) and may have lost its prior to the assay run. Other prenyltransferases purified from Saccharomyces cerevisiae were shown to be very unstable and half-life was shown to be approximately 15 days (Eberhardt and Rilling, 1974). Indeed, Ihara et al. effectively purified the transferase enzyme 6,800 fold from crude extract, but it was very unstable and lost most of the activity in a day. Because of this, assays were subsequently performed within 48 hours with freshly harvested yeast.

Attempts at increasing protein concentration prior to enzyme assays also failed (DMAPP:5'-AMP transferase assay 4) to detect transferase reaction products. R.O. Morris (personal comm.) has suggested that the DMAPP:5'-AMP transferase is membrane bound, very unstable, and denatures

very easily. Assays using Triton X 100 (modified lysis buffer) to solubilize membrane-bound proteins failed to result in a test positive for DMAPP:5'-AMP transferase reaction products (data not shown). Additional attempts at increasing protein stability during enzyme purification, via addition of glycerol (Gekko and Timashoff, 1981), failed to help in the detection of transferase reaction products. Furthermore, the addition of appropriate protease inhibitors in preparation of crude extracts did not aid in the detection of transferase products in S. cerevisiae, although tests performed after each purification step showed that endoproteolytic inhibition was effective (data not shown). In fact, the only enzyme assays to detect DMAPP:5'-AMP transferase reaction products were the ones that contained the cloned Ti plasmid transferase gene (E. coli strain HB101-pTHH21 which contains the ipt region) and represented a high level of transferase. Hence, transferase reaction products in yeast are below the level of detection using standard assay techniques.

Addition of DMAPP and 5'-[2-^{3}H]AMP to cell-free extracts of HB101 (pTHH21) resulted in the incorporation of radioactivity into cytokinins (DMAPP:5'-AMP transferase assay 6). The preparation of enzyme extracts for yeast and HB101-pTHH21 were identical, and run under identical assay conditions, yet only the HB101 strain showed transferase reaction products (Figure 21, lane 1). Thus, our assay

parameters were reliable for detection of transferase reaction products.

DMAPP:5'-AMP transferase products were also detected in HB101-pTHH21 fractions obtained before (Figure 22, lane 2) and after the DEAE purification step (Figure 22, lane 4). When DMAPP was not added as a substrate, there were no transferase products detected (Figure 22, lanes 3 and 5). No DMAPP:5'-AMP transferase reaction products were detected in the S. cerevisiae assays, either before or after the DEAE column (Figure 22, lanes 5-9). Addition or subtraction of DMAPP had no effect in the yeast transferase assays (Figure 22, lanes 5-9). There also seemed to be additional reaction products in the assays run after the DEAE column in both HB101 (pTHH21) and 1480 regardless of whether DMAPP was added.

The level of detection and lability of DMAPP:5'-AMP transferase was tested in DMAPP:5'-AMP transferase assay 8. Addition of DMAPP and 5' [2-^{3}H] AMP to three-week old cell-free extracts of E. coli HB101 resulted in the incorporation of radioactivity into cytokinins. It was found that six week crude homogenate contained no detectable transferase reaction products. Therefore, the crude homogenate is very labile and the ability to detect transferase products is lost between three and six weeks after ion-exchange chromatography. Furthermore, as little as 1 ml HB101 (pTHH21) crude homogenate (1.8 mg protein/ml) showed

detectable transferase products.

Although purification by DEAE cellulose ion-exchange chromatography produced fractions with detectable enzyme products, the second half DEAE eluent fractions were only able to catalyze incorporation of radioactivity into iPAdo, not iPAde. Hence, the first fractions that came off the ion-exchange column were more active, although both the first and second half DEAE fractions contained transferase products.

Importantly, S. cerevisiae seemed to inhibit production or detection of DMAPP:5'-AMP transferase products, but not below the level of detection when mixed with extracts from HB101 (pTHH21) (DMAPP:5'-AMP transferase assay 10). Mixing extracts from HB101 (pTHH21) with extracts from S. cerevisiae resulted in the production of radioactive iPAdo and iPAde (Figure 25), but at lower levels than when HBB101 (pTHH21) was assayed alone. The S. cerevisiae strain alone failed to show any reaction products. The S. cerevisiae strain seemed to inhibit production or detection of iPAdo and iPAde. Interestingly, bead disruption of extracts seemed to produce more inhibition/mg protein than toluene lysing of cell extracts. If S. cerevisiae is indeed producing an inhibitor, it is probably not of low molecular weight, since all extracts were extensively dialyzed prior to assaying.

Free cytokinins in S. cerevisiae are present at

exceedingly low levels (Laten and Zahareas-Doktor, 1985). Indeed, the best preparations of the enzyme that have been obtained to date synthesize only 10 fmol iPAdo-5'-monophosphate/mg protein/hour (Akiyoshi and Morris, unpublished). The relatively weak intensity of the iPAdo and IPAde TLC spots from HB101 (pTHH21) represent a relatively high amount of enzyme reaction products. If HB101 (pTHH21) depicts a high level of enzyme products, the expected amount of transferase reaction products from S. cerevisiae are probably too low to detect using standard assay techniques.

Even under optimal assay conditions, the transferase reaction products in S. cerevisiae are probably below the level of detection. Although my assay parameters were reliable for detection of transferase products, I cannot rule out the possibility that I did not have the correct assay conditions specific for detection of reaction products in S. cerevisiae. The reaction conditions may have lacked a proper divalent cation, or the transferase reaction may be using Ade, not 5'AMP as one of the substrates. The reaction may also be using a separate 5 carbon pyrophosphate other than DMAPP. Assay to assay variability may also have contributed to low reaction products. An alternative hypothesis is that there is a separate, unknown pathway for production of DMAPP:5"AMP transferase products.

Although the data suggests low reaction products in

the S. cerevisiae strain, there is no basis to change any assay conditions. The greatest problem is that enzyme levels are extremely low. An attempt to isolate transferase enzyme from protease deficient yeast using the same parameters is a possibility, or the parameters for yeast tRNA transferase could be employed. Greater amounts of yeast could be used in combination with a specific purification procedure such as affinity chromatography. Affinity chromatography with one of the substrates attached (either 5'AMP or DMAPP) has the possibility of being much more specific for isolation of the putative transferase enzyme. Anti-iPAdo-transferase antibodies have yet to be developed, and even if they were made against A. tumefaciens transferase, it is highly unlikely that they would be an aid in isolating yeast transferase because transferase genes are evolutionarily different even in closely related species (Morris, 1986).

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