



1986

## Metabolism of N<sup>6</sup>- $\Delta^2$ -Isopentenyladenine in *Saccharomyces Cerevisiae*

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METABOLISM OF N<sup>6</sup>- $\Delta$ -2-ISOPENTENYLADENINE  
IN SACCHAROMYCES CEREVISIAE

by

Carol Anne Van Kast

A Thesis Submitted to the Faculty  
of the Graduate School of Loyola  
University of Chicago in Partial  
Fulfillment of the Requirements  
for the Degree of  
Master of Science

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1986



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## VITA

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- Laten, H.M., Doktor, S.Z., Tonetti, D.A., Quinn, M.,  
Timmons, R.M., and VanKast, C.A. (1984) Synthesis and  
Metabolism of Isopentenyladenosine in Yeast. Abstr.  
Annu Meet Am Soc Microbiol. 84 (0), 1984 Abstract H158.
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cerevisiae J. Bacteriol. (in press).

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## Purpose

Cytokinins, a group of N<sup>6</sup> substituted adenine derivatives, are best known for their hormonal functions in higher plants. However, by nature of their presence in certain tRNA species they exist in the cells of nearly all organisms. In order to elucidate possible functions of cytokinins in systems other than plants our lab is exploring the synthesis, metabolism and role of cytokinins in the yeast Saccharomyces cerevisiae.

The purpose of this thesis is to determine whether or not S. cerevisiae can metabolize cytokinins, and if so, the identity of the metabolites.

## INTRODUCTION

### Cytokinin Function

#### Plant Systems

Isopentenyladenosine and other cytokinins are best known for their hormonal functions in higher plants. They have powerful effects on growth and development at every stage of ontogeny.

Cytokinins were first isolated on the basis of a requirement for growth of plant cells demonstrated in excised tobacco pith tissue (Jablonski and Skoog, 1954). In these experiments, when auxin alone was supplied, the cells enlarged, but did not divide: thus illustrating the need of cytokinins as well as auxins for growth and viability of plant tissues.

Cytokinins have been shown to have some influence over tissue differentiation, the initiation of organ primordia, and organ development. In many cases, it is the ratio of cytokinin to auxin in a particular area of a plant which determines which organs are to be formed. For example, a high cytokinin:auxin ratio promotes bud formation and inhibits root formation. On the other hand, a high

auxin:cytokinin ratio will promote roots and inhibit budding (Skoog and Schmitz, 1979).

Cytokinins have also been shown to affect leaf growth. In leaves it is the cytokinin:gibberellin ratio which is critical for development. A high cytokinin:gibberellin ratio results in round shaped leaves. As the ratio is lowered, the leaves take on a spindly, needle-like appearance (Engelke et. al. 1973).

Thimann and Wickson (1957) demonstrated that exogenously supplied cytokinins release lateral buds in Pisium from apical dominance. Studies by Davidson (1971) demonstrated the quantitative relationship between cytokinin dosage applied to lateral buds in peas and the rate of the bud's development and its effectiveness in suppressing growth of the terminal shoot. When cytokinins are applied, the auxin level in the lateral bud rises. At this time auxin production by the terminal bud is suppressed. Ultimately, the laterals completely shut down the auxin production of the terminal shoot and develop into main shoots themselves. This release of lateral buds from apical dominance may be accomplished by the mobilization of nutrients to the growing parts by the cytokinins.

Cytokinins, in conjunction with auxins, increase respiration rates sufficiently to sustain continuous growth in excised tissue cultures (E.H. Newcomb, unpublished). They may do this by promoting the formation of starches which

serve as an energy source (Thorpe and Murashige, 1968). Although a nutritional role for starch is most likely, a possible role in osmoregulation has not been ruled out.

In senescing tissue cytokinins depress respiration as found by Richmond and Lang (1957). This reduction in respiration aids the plant in its maintenance of normal protein levels and slows the aging process. Although it appears that cytokinins can regulate protein levels in senescing tissues it is uncertain whether protein levels are maintained by the inhibition of protein degradation or by an increase in protein synthesis. Kuriashi (1976) and Tavares and Kende (1970) found evidence to support the hypothesis of cytokinin inhibition of polypeptide degradation. Kuriashi found that radiolabelled amino acids incorporated into protein before treatment with kinetin were retained by tobacco tissue but he found no evidence of a kinetin-induced increase in protein synthesis. Tavares and Kende showed that benzylaminopurine in corn leaves retarded degradation but did not increase protein content. Other studies, by Atkin and Srivastava (1970) and Jouanneau and Peaud-Le Noil (1967), indicate that cytokinins stimulate protein synthesis by demonstrating an increase in the incorporation of radioactive amino acids when cytokinins are applied. A study by Takegami (1975a,b) demonstrated both inhibition of protein degradation and stimulation of polypeptide synthesis by a cytokinin. He showed that the incorporation of [ $^{14}\text{C}$ ]

leucine was enhanced by benzylaminopurine and that in the presence of an inhibitor of leucine incorporation protein levels were maintained by benzylaminopurine. It's still uncertain at which stage of protein synthesis cytokinins exert their effect.

Cytokinin treatment has been shown to increase the levels of both DNA and RNA. As in the case of proteins, investigators are divided on the issue of whether cytokinins inhibit nucleic acid degradation by nucleases or stimulate DNA and RNA synthesis.

#### Nonplant Systems

Cytokinins are present in the cells of virtually all organisms as one of many base modifications present in tRNA. These tRNA-associated cytokinins appear to enhance translation and increase the stability of the tRNA-mRNA-ribosomal complex. These functions of tRNA-associated cytokinins have been documented in E. coli by Gefter and Russel (1969) and by Faulkner and Uziel (1971). They have also been observed in yeast by Fittler and Hall (1966) and Laten et. al. (1980).

In addition to these tRNA functions cytokinins have been observed to affect DNA and RNA synthesis in nonplant systems. In higher plants isopentenyladenosine has a positive stimulatory effect on nucleic acid synthesis. In mammalian cells, isopentenyladenosine was found to have

stimulatory or inhibitory effects depending upon its concentration and the stage of the cell cycle during which it was applied (Gallo et. al. 1969, 1972).

In these experiments human leukocytes and lymphocytes were treated with phytohemagglutinin (PHA) to transform them into dividing cells. If isopentenyladenosine is added during the next 20 hours, during  $G_0$  and  $G_1$ , its effects are inhibitory with regard to DNA synthesis, mitosis, and transformation. If isopentenyladenosine is added while the cells are in S phase, after 24 hours, it can stimulate these processes or inhibit them depending upon its concentration. Concentrations of isopentenyladenosine which inhibit DNA synthesis, mitosis, and transformation were found to be greater than  $10^{-6}$  M, while concentrations between  $10^{-7}$  and  $10^{-6}$  were stimulatory.

The inhibitory effect observed when isopentenyladenosine was added during growth phases seems to be due to an inhibition of the synthesis of new RNA as opposed to the inhibition of DNA or protein synthesis. DNA synthesis does not occur until S phase and isopentenyladenosine added at this phase can have a stimulatory effect. The only proteins inhibited were those that were newly synthesized after PHA was added - protein formation that occurs in these cells without the addition of PHA was unaffected. Therefore, it seems that isopentenyladenosine inhibition occurs at the level of RNA synthesis.

Isopentenyladenosine, in preliminary studies, has been observed to have some modest anti-tumor properties in humans. It inhibits the growth of tumor cells in vitro (Fleysher, 1972) and in vivo (Suk et. al., 1970). In preliminary studies, Mittleman et. al. (1975) found isopentenyladenosine to be able to induce remissions in leukemia patients.

In yeast, the presence of isopentenyladenosine has been established in tRNA (Zachau et.al., 1966; Madison et. al. 1967; and Hecht et. al., 1969) and in free form (Laten and Zahareas-Doktor, 1984). While the function of yeast tRNA associated cytokinins has been elucidated by Gefter and Russel (1969) and Laten et. al. (1980), the function of free isopentenyladenosine has yet to be established. Laten et. al. (unpublished) have hypothesized that there is no straightforward role for cytokinins in yeast cell growth and division. They found that the growth rate of wild type Saccharomyces cerevisiae was unaffected by various concentrations of several common cytokinins added to the growth medium. However, cytokinin ribosides were used in these experiments. Later experiments suggested that cytokinin ribosides do not enter the S. cerevisiae cell. Analogous experiments with the cytokinin bases have yet to be conducted.

### Chemical Structure and Activity

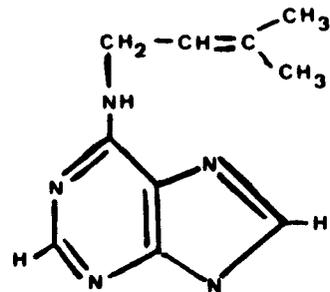
Virtually all naturally occurring cytokinins are derivatives of adenine. Figure 1 contains examples of common, naturally occurring cytokinins. In all monosubstituted adenines known to possess cytokinin activity the substituent is present at the N<sup>6</sup> position. If the substituent is elsewhere on the purine double ring the effect of these substituted adenines on cell division can be inhibitory (Szwedowska and Korcz, 1972).

The nature of the substituent attached at the N<sup>6</sup> position has profound effects on cytokinin activity. Cytokinin activity is assessed by the ability of this compound to promote the growth (increase in fresh weight) of a cytokinin-dependent tobacco or soybean callus (Linsmaier and Skoog, 1965).

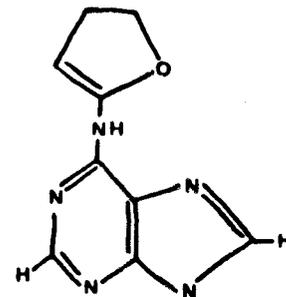
Cytokinin activity increases with an increase in the chain length of the substituent and is maximized at five carbons. Activity decreases tenfold with each addition or subtraction of substituent carbon atoms. The presence of unsaturation in the N<sup>6</sup> substituent has been shown to markedly increase activity. Also important is the location of the double bond. The unsaturated  $\Delta^2$ -isopentenyl group of isopentenyladenine is the parent side chain of high potency cytokinins, conferring about ten times higher activity than an isopentyl or n-pentyl group (Skoog *et. al.*, 1967). If the double bond of the isopentenyl group is moved

Figure 1.

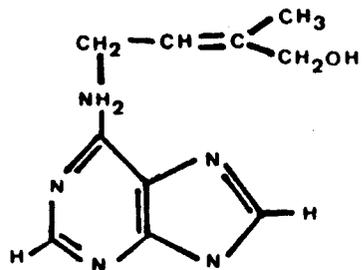
Structures of some naturally occurring cytokinins.



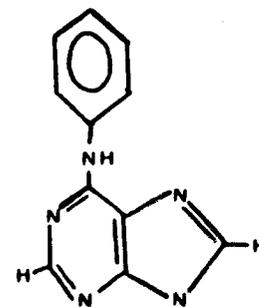
**N<sup>6</sup>-(Δ<sup>2</sup> ISOPENTENYL)ADENINE**



**KINETIN**



**ZEATIN**



**6-BENZYLAMINO PURINE**

from the second carbon of the side chain to the third, a decrease in activity is observed (Leonard *et. al.*, 1968). The presence of hydroxyl groups on the N<sup>6</sup> substituent also influences activity. Their presence on the fourth carbon of the side chain increases activity, while their presence at the second or third carbon tends to inhibit activity (Leonard *et. al.*, 1969). Zeatin, the most potent natural cytokinin, incorporates the most effective characteristics in the side chain - unsaturation in the second position and hydroxylation at the terminal end.

#### Cytokinins in tRNA

Cytokinins, by virtue of their presence in certain tRNA species are present in the cells of virtually all organisms. Cytokinins appear in tRNA as one of many base modifications. Isopentenyladenosine exists in the tRNA of all organisms thus far investigated. The cytokinin *cis*-zeatin is predominant in plant tRNA (Skoog and Schmitz, 1979).

All tRNA species which possess cytokinin activity respond to codons in which the first base in the 3'-5' direction is uridine. In these tRNAs, the modified nucleoside which is the cytokinin, is always the monosubstituted adenosine located in the position adjacent to the 3' end of the anticodon. This modified adenosine is always in the middle of a triple adenosine sequence.

In E. coli, cytokinins exist in all tRNAs responding to codons starting with uridine (Armstrong et. al., 1969). This is not the case in most organisms, however. In yeast, tRNAs responding to codons for serine, tyrosine, and cysteine have been shown to contain cytokinin activity. Yeast serine, tyrosine and cysteine were found to possess cytokinins by Zachu et. al. (1966), Madison et. al. (1967), and Hecht et. al. (1969), respectively. In all animal cells thus far examined the presence of cytokinins has only been detected in serine tRNAs (Skoog and Armstrong, 1970). In the tRNA of higher plants cytokinins have been found in serine and leucine tRNA (Skoog and Schmitz, 1979).

The function of tRNA-associated cytokinins appears to be the enhancement of translation. They also seem to improve the efficiency of tRNA binding to the mRNA-ribosomal complex (Fittler and Hall, 1966).

Altering or removing the N<sup>6</sup> substituent on the adenosine can markedly affect the ability of the tRNA to function in protein synthesis. When phenylalanine tRNA from E. coli was treated with iodine, the tRNA was unable to bind to the mRNA-polyribosomal complex - a phenomenon due to the reaction of the iodine with the cytokinin moiety (Faulkner and Uziel, 1971). Laten et. al. (1980) isolated tRNA from a mutant strain of yeast which had 2% of the normal complement of isopentenylated tRNA. It was found to accept normal

levels of amino acids in vitro, but in vivo its function as a suppressor tRNA was markedly impaired.

The importance of the nature of the N<sup>6</sup> substituent for protein synthesis was demonstrated in a study by Gefter and Russell (1969). From E. coli, they purified three strains of suppressor tyrosine tRNA which differed only in the N<sup>6</sup> substituent on the adenosine in the position adjacent to the 3' end of the anticodon. Species A contained an unmodified adenosine. Species B had an isopentenyladenosine, and species C a 2-methylthio-isopentenyladenosine. Aminoacylation was unaffected, but binding of the tRNA to the mRNA-ribosome complex and protein synthesis were strongly affected by the nature of the N<sup>6</sup> modification. Unmodified adenosine was least effective at binding to the ribosomal complex and was not able to support protein synthesis. 2-methylthio-isopentenyladenosine was very effective in binding to the ribosome and in polypeptide synthesis. Isopentenyladenosine was intermediate in its ability to bind to the mRNA-ribosomal complex and to sustain protein synthesis.

Although some knowledge has been gained with respect to cytokinin function on the molecular level, it is still not possible to relate the function of cytokinins in protein synthesis to their role as growth hormones.

## Cytokinin Biosynthesis

### tRNA-associated Cytokinins

The synthesis of a tRNA-cytokinin association begins with mevalonate, an intermediate in the sterol biosynthetic pathway. Mevalonate is converted to  $\Delta^3$ isopentenylpyrophosphate, which is then isomerized to the  $\Delta^2$  form.  $\Delta^2$ isopentenylpyrophosphate (iPP) donates its isopentenyl group to the adenosine molecule adjacent to the 3' end of the anticodon of an already formed tRNA molecule.

That mevalonate is the precursor of the side chain of this tRNA-associated cytokinin was established by Fittler *et. al.*, (1968); Peterkofsky, (1968); and by Chen and Hall, (1969) on Lactobacillus acidophilus, L. plantarum, and tobacco, respectively. When  $^{14}\text{C}$  mevalonate was supplied to the mevalonate-requiring bacteria and to tobacco pith culture, the tRNA became labelled. All of the label was accounted for by the isopentenyl group of isopentenyladenosine.

The fact that the isopentenyl group attaches to preformed molecules was demonstrated by experiments by Fittler *et. al.*, (1968b) and Chen and Hall, (1969). These investigators isolated an enzyme from yeast, rat liver, and tobacco pith which catalyzed the transfer of the isopentenyl group from isopentenylpyrophosphate to tRNA. This enzyme was shown to catalyze the transfer of  $^{14}\text{C}$  isopentenyl-

pyrophosphate to tRNA which had previously been treated with permanganate to remove the isopentenyl side chain.

### Free Cytokinins

The synthesis of free cytokinins has been shown to occur by two pathways 1) from the breakdown of cytokinin-containing tRNA, and 2) de novo synthesis independent of tRNA. The method used seems to depend on the organism which is being examined.

In plants, both methods are likely for free cytokinin biosynthesis. Plant tRNA has been shown to contain cytokinins, so the release of isopentenyladenosine and other cytokinins upon tRNA breakdown seems likely. However, the level of cytokinins in higher plants is such that it can not be accounted for solely by the turnover of cytokinin-containing tRNA. Short and Torrey (1972) showed that the free cytokinin activity of pea root tips is 27 times the activity present in the tRNA. Furthermore, if tRNA hydrolysis is responsible for free cytokinin activity, then other tRNA associated cytokinins such as cis-zeatin ribose would be present as free cytokinins. None have been thus far detected except as tRNA base modifications (Letham, 1978). Chen and Hall (1969) and Burrows (1976) found that cytokinin-auxotrophic tissue had cytokinins in their tRNA. The existence of tissues that have tRNA associated cytokinins and yet require an exogenous supply of cytokinins

indicates that tRNA turnover does not yield an adequate supply and that cytokinin autonomous plant tissues must utilize at least one other method of cytokinin biosynthesis.

In yeast, Laten and Zahareas-Doktor (1984) established the presence of a tRNA-independent biosynthetic pathway for isopentenyladenosine. By using a radioimmunoassay, they determined that the levels of free isopentenyladenosine in yeast cells deficient in tRNA-associated isopentenyladenosine were at least as great as those in equivalent wild type strains of Saccharomyces cerevisiae and Schizosaccharomyces pombe.

In some bacteria tRNA turnover has been shown to be adequate for the level of free cytokinins found. Hahn et. al., (1976) found that the amount of isopentenyladenine in Agarobacterium tumifaciens can be accounted for by tRNA turnover. Similar evidence was compiled in Lactobacillus acidophilus by Helback and Klemmt, (1978).

Methods of de novo synthesis have been elucidated in several organisms. Taya et. al. (1978) demonstrated that Dictostelium discoidium formed isopentenyladenine from isopentenyladenosine monophosphate, which arises from iPP and AMP. Chen and Melitz (1979) also found an enzyme system which catalyzes the formation of isopentenyladenosine monophosphate from iPP and AMP. In cruder enzyme preparations isopentenyladenosine and isopentenyladenine were also detected, indicating the presence of enzymes to

convert isopentenyladenosine monophosphate to isopentenyladenine and isopentenyladenosine. The same sequence of events was found by Murai (1981) to be present in Corynebacterium fascians.

### Cytokinin Metabolism

#### Plant Systems

The metabolism of cytokinins in higher plants can occur via several pathways. First, a cytokinin free base may be converted to a riboside and/or a nucleotide. Second, the cytokinin may have its side chain modified or removed. Third, the purine moiety may be modified.

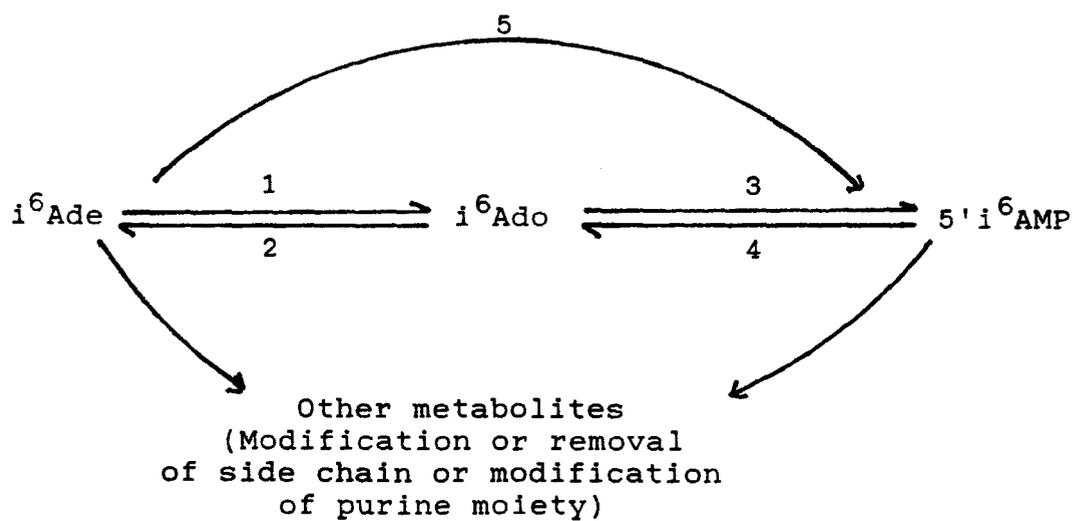
Most of the enzymes involved in the interconversion of isopentenyladenine, isopentenyladenosine, and isopentenyladenosine monophosphate were identified by Chen and coworkers (Fig 2.). Chen and Petschow (1978) demonstrated the conversion of isopentenyladenine to isopentenyladenosine via the adenosine phosphorylase of wheat germ. Chen and Kristopeit (1981) identified the adenosine nucleosidase which catalyzes the reverse reaction. Chen and Eckert (1977) detected the activity of an adenosine kinase which converts isopentenyladenosine to isopentenyladenosine monophosphate in wheat germ. The reverse reaction is carried out by a 5'-nucleotidase observed by Chen and Kristopeit (1981).

Similar pathways can be applied to zeatin and benzylaminopurine metabolism. In lupins, Summons et. al.

**Figure 2**

The metabolism of cytokinins in wheat germ, according to Chen and coworkers.

1. Adenosine phosphorylase, 2. Adenosine nucleosidase,
3. Adenosine kinase, 4. 5'nucleotidase, 5. Adenine phosphoribosyltransferase.



(1981) found that zeatin could have its side chain reduced forming dihydroxyzeatin, and that both zeatin and dihydroxyzeatin could be converted to ribosides and nucleotides. Benzylaminopurine was shown by Fox et. al., (1972) to be metabolized to the ribonucleoside and ribonucleotide in soybean callus.

Cytokinins can also be metabolized by the cleavage of the N<sup>6</sup> side chain. This is a common fate for exogenously supplied cytokinins and is thought to be one way a plant maintains a constant level of cytokinins. The removal of a side chain is carried out by a cytokinin oxidase. Cytokinin oxidase was isolated from Zea mays by Whitty and Hall (1974) and from tobacco by Paces et. al., (1971). Substrates for this enzyme include isopentenyladenine, isopentenyladenosine, zeatin and zeatin ribose. Benzylaminopurine is not a substrate due to the presence of a benzene ring on the N<sup>6</sup> substituent. Dihydrozeatin is also unaffected by the enzyme, illustrating the need of a double bond in the substituent for activity (McGaw and Horgan, 1983).

An additional metabolic option open to plants is to form cytokinin glucosides. Isopentenyladenine, zeatin and benzylaminopurine have all been shown to form 7-glucosides. They were identified by Laloue et. al. (1975, 1977) in tobacco, by Parker and Letham (1973) in radish seedling cotyledons, and by Fox (1972) in soybeans, respectively.

It seems that cytokinin bases are the only forms which are converted to the glucoside. This was first observed by Laloue et. al. (1975, 1977) in tobacco where they showed that isopentenyladenosine is converted to isopentenyladenosine monophosphate, its diphosphate and triphosphate forms, while isopentenyladenine is converted into the 7-glucoside. In soybeans, Fox (1972) observed that benzylaminopurine formed its nucleoside and nucleotides first upon entering soybean callus. Later it was converted to benzylaminopurine-7-glucoside. Fox also noted that the benzylaminopurine nucleosides and nucleotides were degraded after five days but the glucoside was still present after 60 days.

It seems that the glucoside group protects the cytokinin from side chain cleavage. The role of this glucoside form appears to be long term storage of cytokinins.

McGaw and Horgan (1983), however, argue that the tissues used in these studies do not have high levels of cytokinin oxidase activity and this is why the 7-glucoside form appears stable. They cite as evidence the low level of cytokinin oxidase catabolism when Parker and Letham (1973) applied zeatin and Letham et. al. (1982) applied zeatin-7-glucoside to raddish tissue. Since zeatin is a substrate of cytokinin oxidase, it should have been rapidly degraded. The fact that it was not led McGaw and Horgan to conclude that

there must be low levels of cytokinin oxidase in raddish tissue and this is why the glucoside appears immune to oxidase activity. They propose that in Zea mays and Vinca rosea the O-glucosides are the stable storage forms of cytokinins.

### Animal Systems

Cytokinins are present in animal cells in the form of tRNA-associated isopentenyladenosine. Isopentenyladenosine is metabolized by these cells via tRNA breakdown followed by conversion of the freed isopentenyladenosine to inosine by an adenosine aminohydrolase enzyme.

Adenosine aminohydrolase enzyme was isolated from calf intestinal mucosa and chicken bone marrow by Hall et. al. (1971). Although the calf intestine enzyme will degrade isopentenyladenosine to inosine it does so at a rate 1000 times slower than it degrades adenosine. The chicken bone marrow enzyme catalyzes the conversion of adenosine to inosine only 40 times faster than the analogous reaction with isopentenyladenosine.

Analogues of isopentenyladenosine were also tried as substrates for the bone marrow enzyme. the  $\Delta^3$  isomer, n-pentyl, and furfural derivatives were all readily cleaved. Hydroxylated derivatives, however, were not found to be substrates of the adenosine aminohydrolase enzyme.

Since the reaction rate of the calf intestinal mucosal enzyme with isopentenyladenosine is so slow, it does not seem likely to be an isopentenyladenosine catabolic enzyme. The bone marrow enzyme, on the other hand, does seem to be a feasible method of isopentenyladenosine degradation. Furthermore, McLennan et. al. (1968) found that a crude extract of human bone marrow is also able to convert isopentenyladenosine to inosine.

### Yeast Systems

The metabolism of cytokinins in yeast appears to occur in two ways. First, yeast, specifically Schizosaccharomyces pombe, has been shown to possess an adenine aminohydrolase which converts N<sup>6</sup> substituted purines to hypoxanthine. Hypoxanthine can then be converted to inosine monophosphate, an AMP biosynthetic intermediate, via hypoxanthine guanine phosphoribosyl transferase (DeGroot et. al., 1969). Second, studies done by Laten et. al. (unpublished), demonstrate that another yeast, Saccharomyces cerevisiae possesses a somewhat different method of metabolism. S. cerevisiae appears to use a cytokinin oxidase-like enzyme to degrade cytokinins.

Abbondandolo et. al. (1971) utilized the yeast Sch. pombe in studies of the metabolism of N<sup>6</sup> substituted purine analogues in this species. Eight adenineless mutants were

selected after the mutagenic treatment of wild type Sch. pombe cells (Fig 3.). When these adenine auxotrophs were grown on media containing 6-chloropurine or one of a series of other N<sup>6</sup> substituted purines, all but two were able to grow, demonstrating their ability to convert these substances to AMP (Table 1.). Abbondandolo was able to demonstrate the conversion of 6-chloropurine to hypoxanthine in vitro using crude cell extracts from the ade-1 strain. From this piece of evidence he proposed that Sch. pombe metabolizes 6-chloropurine and other N<sup>6</sup> substituted purines to hypoxanthine and that hypoxanthine is converted to IMP via hypoxanthine guanine phosphoribosyl transferase. The IMP is then converted to AMP by the last two enzymes in the AMP biosynthetic pathway. Abbondandolo et. al. (1971) also hypothesize, based on mutant studies, the existence of two genes to control adenine aminohydrolase; one of them being the structural gene, the other a regulator gene.

Guern et. al. (1972), in experiments similar to those of Abbondandolo et. al., (1971) demonstrated the ability of Sch. pombe to convert cytokinins to hypoxanthine. In these experiments the cytokinins used as substrates were isopentenyladenine, zeatin, benzylaminopurine and kinetin. Guern et. al. (1972) also used an in vitro assay to establish in cell extracts of Sch. pombe the presence of adenine aminohydrolase. The differences between Guern's

Figure 3.

The metabolism of cytokinins in Sch. pombe, according to Abbondandolo et. al. (1971).

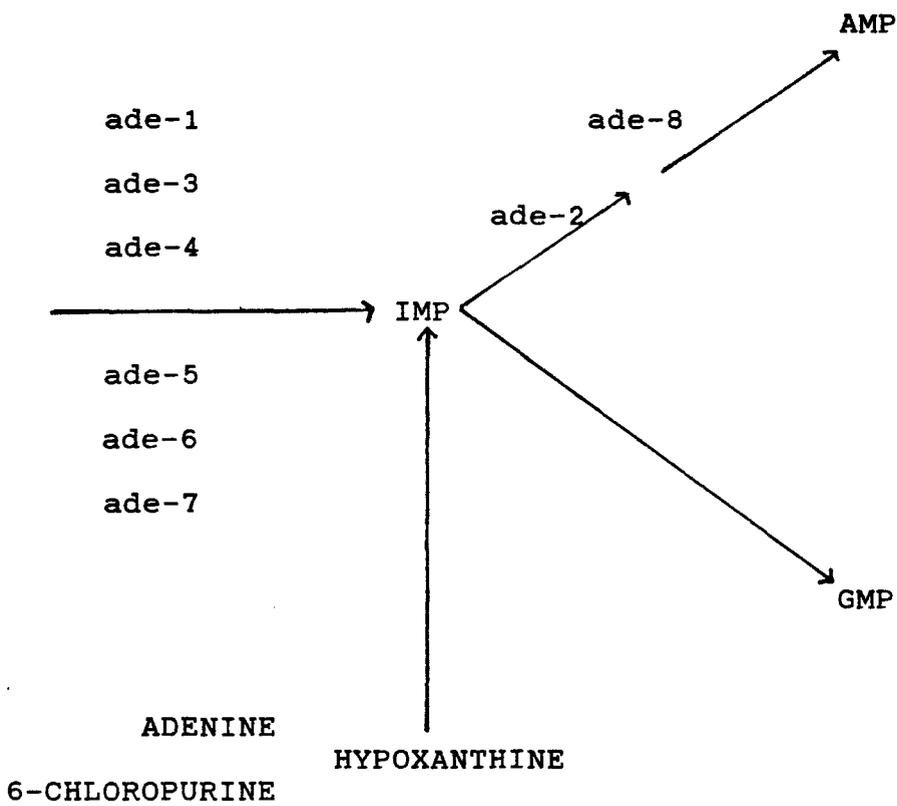


Table 1.  
Growth of adenine auxotrophs of S. pombe on N<sup>6</sup>-substituted  
purines.

Supplement	ade-1	ade-2	ade-3	ade-4	ade-5	ade-6	ade-7	ade-8
Adenine	+	+	+	+	+	+	+	+
Hypoxanthine	+	0	+	+	+	+	+	0
6-Methoxypurine	+	0	+	+	+	+	+	0
6-Ethoxypurine	+	0	+	+	+	+	+	0
6-propoxypurine	+	0	+	+	+	+	+	0
6-Butoxypurine	+	0	+	+	+	+	+	0
6-Methyl mercaptopurine	+	0	+	+	+	+	+	0
6-Ethyl mercaptopurine	+	0	+	+	+	+	+	0
6-Propyl mercaptopurine	+	0	+	+	+	+	+	0
6-Carbaethoxy methyl mercaptopurine	+	0	+	+	+	+	+	0
6-Chloropurine	+	0	+	+	+	+	+	0
6-Iodopurine	+	0	+	+	+	+	+	0
6-Carboxypurine	+	0	+	+	+	+	+	0
6-Cyanopurine	+	0	+	+	+	+	+	0
6-Dimethyl aminopurine	+	0	+	+	+	+	+	0
6-n-Hexyl aminopurine	+	0	+	+	+	+	+	0

study and that of Abbondandolo was that Guern used N<sup>6</sup> substituted compounds known to possess cytokinin activity in his assay. Also, he used extracts from wild type Sch. pombe as opposed to adenine auxotrophs.

The experiments discussed in the present study include an in vivo study similar to that of Abbondandolo et. al., (1971). Adenineless strains of S. cerevisiae were grown on cytokinin supplemented media to establish the existence of a cytokinin oxidase in this yeast. The observation that mutants with blockages after IMP in the AMP biosynthetic pathway were able to grow when given a cytokinin instead of adenine indicated the presence of an enzyme system distinct from adenine aminohydrolase.

These experiments were taken one step further by attempts to grow these adenineless cells on media containing radioactive isopentenyladenine. The intention was the identification of radioactive products of isopentenyladenosine metabolism. As we were unable to accomplish this goal, we attempted to demonstrate S. cerevisiae cytokinin metabolism in vitro. Crude cell extracts of wild type S. cerevisiae were assayed for the ability to convert radioactive isopentenyladenosine to metabolic products. Products were detected in the assay, but their identity is presently unknown.

## Materials and Methods

### Cytokinin Metabolism in vivo

Yeast strains used were adenine-requiring mutants of S. cerevisiae and Sch. pombe and are listed in Table 2. The strains were grown on synthetic dextrose (SD) minimal medium (2% dextrose, 0.67% yeast nitrogen base) with 2% agar and 1% of each required amino acid. The experimental medium was supplemented with cytokinins or adenine, and the control medium contained no supplements.

Supplements used were isopentenyladenine, isopentenyladenosine, kinetin, kinetin ribose, zeatin, zeatin ribose, benzylaminopurine, benzylaminopurine ribose, dihydroxyzeatin, hypoxanthine, inosine, adenine and adenosine. The concentration used in all cases was 0.02%.

Suspensions of the yeast strains in water were added to the control and supplemented plates and checked for growth after four days at 30 °C.

### Synthesis of [<sup>3</sup>H]N<sup>6</sup>- $\Delta^2$ -Isopentenyladenosine

N<sup>6</sup>- $\Delta^2$ -isopentenyladenosine was synthesized by the method of Paces et. al. (1971). 600  $\mu$ Ci of [2,5,8-<sup>3</sup>H]-adenosine with a specific activity of 41 Ci/mmole were dissolved in 10 ml of water. The solution was rotoevaporated to dryness. The residue was resuspended in 2 ml of absolute

Table 2.

Mutant strains of S. cerevisiae used in the in vivo cytokinin metabolism study.

Table 2.

<u>Species</u>	<u>Strain</u>	<u>Enzyme</u>
<u>Sch. pombe</u>	ade-1	Glycinamide ribonucleotide synthetase Aminoimidazole ribonucleotide synthetase
<u>S. cerevisiae</u>	ade-2	PR-aminoimidazole carboxylase (5'-PR-5-amino-4-imidazolecarboxylate carboxylase, 4.1.1.21)
<u>S. cerevisiae</u>	ade-4	amido-PR-transferase (5-PR-amine: pyro-P PR-transferase glutamate-amidating, 2.4.2.14)
<u>S. cerevisiae</u>	ade-5	PR-glycinamide synthetase (5-PR-amine: glycine ligase ADP forming 6.3.4.13)
<u>S. cerevisiae</u>	ade-6	PR-formylglycinamide synthetase (5'-PR-formylglycinamide: L-glutamine amidoligase ADP-forming, 6.3.5.3)
<u>S. cerevisiae</u>	ade-12	adenosuccinate synthetase (IMP: L-aspartate ligase GDP-forming, 6.3.4.4)

ethanol and re-evaporated twice. The residue was combined with 5.34 mg of unlabelled adenosine and dried overnight over phosphorous pentoxide. Four microliters of  $\Delta^2$ -isopentenyl bromide and 70  $\mu$ l of dimethylformamide were added to the adenosine. The  $\Delta^2$ -isopentenyl bromide and the dimethylformamide had previously been refluxed for at least 13 hours each. The radioactive mixture was stirred overnight and 70  $\mu$ l of dimethylamine and 100  $\mu$ l of methanol were added and again stirred overnight. The mixture was then dried under a stream of nitrogen gas.

#### Product Assessment After Isopentenylation Reaction

Two methods were employed to quantify the amount of isopentenyladenosine produced by the above reaction.

#### Thin-layer Chromatography

Before the radioactive solution was subjected to thin-layer chromatography (TLC) a nonradioactive separation was done. Ten 1  $\mu$ l aliquots of standard solutions of adenine, adenosine, isopentenyladenine, isopentenyladenosine, and a mixture of all four were spotted on a silica gel TLC plate. The concentration of all standard solutions used was 50 OD/ml (concentration was determined at  $\lambda_{\text{max}}$ ). The solvent system used was ethanol:0.1 M aqueous  $(\text{NH}_4)_3\text{BO}_3$ , pH9 (1:9). The chromatogram was developed for three hours.

In the radioactive separation the standards used were the same as above, but to the mixture of all four standards was added an aliquot (33,200 C.P.M.) of [ $^3\text{H}$ ] isopentenyladenosine reaction solution. The spotting, solvent solution and development time were the same as described above. After development of the chromatogram the radioactive lane was cut out and cut into horizontal pieces, leaving all ultra-violet absorbing spots intact. The pieces were then placed in scintillation vials containing 1 ml of 2 N sodium hydroxide. The vials were shaken gently at room temperature for 90 minutes in a shaker bath. One half ml was removed from each vial and placed in a second scintillation vial. Five milliliters of a toluene-based scintillation cocktail was added to each and the presence of radioactivity in each vial was assessed by liquid scintillation counting.

#### Sep-Pak Separation

One-and-a-half milliliters of a nonradioactive mixture of isopentenyladenosine (2.205  $A_{269}/\text{ml}$ ) and adenosine (4.920  $A_{260}/\text{ml}$ ) was fractionated on a prewetted Sep-Pak cartridge. The cartridge was eluted with five 1 ml aliquots of water, followed by ten 1 ml aliquots of 15% acetonitrile, and ten 1 ml aliquots of 100% acetonitrile. The absorbance of each aliquot was measured at 260 nm and 269 nm, the neutral  $\lambda_{\text{max}}$  for adenosine and isopentenyladenosine, respectively.

The radioactive reaction solution containing [<sup>3</sup>H]-isopentenyladenosine and unreacted [<sup>3</sup>H]-adenosine was loaded on a Sep-Pak cartridge. The Sep-Pak was eluted, one milliliter at a time, with 5 ml of water, 5 ml of 15% acetonitrile, and 5 ml of 100% acetonitrile. A small aliquot of each fraction was counted by liquid scintillation counting.

#### Conversion of Isopentenyladenosine to Isopentenyladenine

The formation of isopentenyladenine from isopentenyladenosine was attempted in three ways. Two methods involved the hydrolysis of the sugar moiety by hydrochloric acid. A third involved the oxidation of a bond within the sugar and the subsequent removal of the group.

#### Hydrolysis of [<sup>3</sup>H]-Isopentenyladenosine to [<sup>3</sup>H]-Isopentenyladenine (1.2 M)

This procedure, reported by Martin and Reese (1968) uses a 1.2 M solution of isopentenyladenosine in 1 N hydrochloric acid. The mixture was heated to 90 °C for one hour. The solution was then neutralized with sodium hydroxide. The salt was removed by elution with 5 ml of water on a Sep-Pak cartridge. The fractions containing isopentenylated compounds were rotoevaporated to dryness and resuspended in methanol for analysis by reverse-phase high performance liquid chromatography (HPLC). An isocratic elution with 55% methanol at a flow rate of 0.5-1.0 ml per

minute was used to identify and purify the isopentenyladenine formed from the hydrolysis. Thin-layer chromatography was also used to verify the presence of isopentenyladenine. The thin-layer was developed under the same conditions as those used in the separation of isopentenyladenosine and adenosine (see above), except that an aliquot of radioactive hydrolysate replaced the aliquot of isopentenylation reaction solution.

#### Oxidation of Isopentenyladenosine to Isopentenyladenine

In the procedure of Chen et. al. (1975), isopentenyladenine was formed by the oxidation of isopentenyladenosine by periodate. A  $1.275 \times 10^{-4}$  M solution of [ $^3\text{H}$ ]-isopentenyladenosine was made. 2 ml of this solution were mixed with 3 ml of 6.67 mM sodium periodate and reacted in the dark at room temperature for 30 minutes, 0.3 ml of a 13.3 mM glucose solution was added, mixed and left at room temperature for 20 minutes, 1.325 ml of 5 N sodium hydroxide was added to make the solution 1 N with respect to sodium hydroxide. The reaction mixture was then boiled in a water bath for 30 minutes. One ml aliquots were taken at time 0, 10 minutes, 20 minutes, and 30 minutes. Each aliquot was neutralized with hydrochloric acid. HPLC was used to analyze the products of the oxidation. Elutions were run using 30% acetonitrile.

Hydrolysis of [<sup>3</sup>H]-Isopentenyladenosine to [<sup>3</sup>H]-Isopentenyladenine (0.015 M)

The second hydrolysis procedure was also reported by Martin and Reese (1968). A 0.015 M solution of nonradioactive isopentenyladenosine in 1 N hydrochloric acid was heated in a 41 °C water bath. Aliquots of 20  $\mu$ l were taken at time 0 and at each hour for 16 hours. 100  $\mu$ l of methanolic ammonia was added to neutralize each aliquot. The methanolic ammonia was made up so that the methanol was half-saturated with ammonium hydroxide at 0 °C.

The aliquots were analyzed using thin-layer chromatography. The aliquots were spotted in ten 1  $\mu$ l quantities on silica gel thin-layer sheets. The solvent system used was ethanol:0.1 M aqueous (NH<sub>4</sub>)<sub>3</sub>BO<sub>3</sub>, pH9 (1:9). Chromatograms were developed for three hours. The spots were cut out and placed in scintillation vials containing 6 ml of 0.1 N hydrochloric acid and shaken gently in a shaker bath at room temperature for 24 hours. The absorbance of each spot was measured at the acidic  $\lambda_{\text{max}}$  for each product; 265 nm for isopentenyladenosine, 273 nm for isopentenyladenine and 272 nm for N<sup>6</sup>-(3-hydroxy-3-methylbutyl)adenine.

In a radioactive run of this procedure a 0.015 M solution of [<sup>3</sup>H]-isopentenyladenosine in 1 N hydrochloric acid was prepared and heated at 41 °C for eight hours. The reaction solution was neutralized with methanolic ammonia. HPLC was used to analyze and purify the radioactive

products. The products were isolated isocratically with either 30% acetonitrile or 55% methanol.

### In vivo Metabolism of [<sup>3</sup>H]-Isopentenyladenine

Table 3 describes the specific conditions of each experiment done in the study of the in vivo metabolism of [<sup>3</sup>H]-isopentenyladenine by S. cerevisiae.

Strains 37 (wild-type), 1500 (ade-12), and 1540 (aah) were used. Precultures were grown in three types of medium; synthetic dextrose (SD) medium containing yeast nitrogen base (YNB) (0.67%) supplemented with 1% adenine, YPD medium (1% yeast extract, 1% peptone, and 2% dextrose), or SD medium with yeast carbon base (1%). Precultures were either 50 ml liquid cultures or 30 ml agar plates and were grown for one day unless otherwise specified. Liquid cultures were grown in a shaker at 300 rpm. Solid agar precultures were grown in a 30 °C incubator.

Growth cultures contained SD medium with either YNB or yeast carbon base (YCB) at a concentration of 1%. In some experiments the media was supplemented with polymixin B nonapeptide (PBN) to aid the uptake of cytokinins. The concentration of PBN used ranged from 0.05 A<sub>260</sub>/ml to 1.0 A<sub>260</sub>/ml. The concentration of isopentenyladenine used varied from 0.001 mM to 1 mM. The amount of radioactive cytokinin used in incorporation studies ranged from 10,000 C.P.M. to 100,000 C.P.M..

Table 3.  
Summary of the in vivo [<sup>3</sup>H]-isopentenlyadenine metabolism  
experiments.

TABLE 3

Expt.	Strains	Preculture	Growth Culture	[ <sup>3</sup> H] C.P.M.	i <sup>6</sup> Ade (mM)	Vol.	Time Points(hrs)
1	1500, 1540	SD + YNB +ade	SD + YNB	90,500	0.1, 0.01, 0.001	1 ml (liq)	0, 4, 8, 25, 50
2	1500	YPD	SD + YNB	0	1.0	50 ml (liq)	0, every 2 h for 28h
3	1500, 154	YPD	SD + YNB	10,000	1.0	1 ml (liq)	0, 4, 8, 29
	1500, 1540	YPD	SD + YNB	0	1.0	1 ml (liq)	0, 4, 8, 29
4	1500, 1540	YPD	SD + YNB	100,000	1.0	1 ml (liq & solid)	0, 48, 72, (liq) 96 (solid)
	1500, 1540	YPD	SD + YNB	0	1.0	50 ml (liq)	0, 48, 72
5	37, 1500 1540	YPD	SD + YCB	0	0.1, 1.0	50 ml (liq)	0, 5, 26
6	37, 1540	YCB (6d)	SD + YCB	0	0.1, 1.0	50 ml (liq)	0, 8, 28, 52, 72.5, 99.5, 122, 145, 176, 196, 222

Expt.	Strains	Preculture	Growth Culture	[ <sup>3</sup> H] C.P.M.	i <sup>6</sup> Ade (mM)	Vol.	Time Points(hrs)
7	1500	YPD plate	SD + YNB + PBN 0, 0.05, 0.1, 0.2, 0.5, 1.0 A/ml	0	1.0	1 ml (liq + solid)	2, 4, 48, 96
8	1500	YPD plate	SD + YNB + PBN 0.2 A/ml	436,000	1.0	0.5 ml (liq)	72

The growth of cultures in the growth cultures was detected by measuring the absorbance of the cultures at 625 nm in a double beam spectrophotometer, or by gross observation on agar growth medium. The incorporation of [<sup>3</sup>H]-isopentenyladenine into the cells was assessed by counting washed cells in a liquid scintillation counter.

### In vitro Assay for Cytokinin Metabolizing Enzymes

#### Cell Preparation

Strain 37 was used to inoculate 50 ml of YPD medium, which served as a preculture. The preculture was incubated overnight at 30 °C in a shaker bath and was used to inoculate 2.2 L of YPD medium. The growth cultures were incubated at 30 °C and 300 rpm and cells were harvested at between 10 and 20 A<sub>625</sub>/ml by centrifugation at 6000 rpm for 20 minutes at 4 °C. The cells were washed once with water and weighed.

#### Protein Extraction

The harvested cells were suspended in toluene in dry ice at a concentration of 50 g of cells per 100 ml of toluene. The suspension was cooled for two hours. The excess toluene was poured off and the frozen cells thawed at 4 °C overnight. Two milliliters of 2 M Tris was added for every 50 g of thawed cells, and the mixture was centrifuged at 16,000

rpm for 20 minutes. The supernatant was then centrifuged at 16,000 rpm for 10 minutes. The supernatant was retained and both pellets discarded.

Streptomycin sulfate was added to a final concentration of 1% to remove nucleic acids. The mixture was stirred in ice for 10 minutes and maintained on ice for eight hours or overnight. The mixture was centrifuged at 10,000 rpm for 20 minutes, and the pellet discarded.

Ammonium sulfate cuts of 0-20%, 20-40%, 40-60%, and 60-80% saturation were used to precipitate proteins. At each level of saturation the mixture was stirred on ice for 10 minutes and centrifuged at 10,000 rpm for 20 minutes.

All ammonium sulfate precipitates were exhaustively dialyzed against 0.1 M sodium phosphate, pH 6.8. Dialyzed protein solutions were concentrated using immersible CX10 ultrafiltration units. Protein concentration was assessed by the method of Lowry et. al. (1951).

### Enzyme Assay

The enzyme assay was run under several different conditions. The assay was done in four different salt environments; 0.04 M sodium phosphate, pH 7 (SP), SP + 0.1 M potassium chloride, SP + 0.01 M magnesium chloride, and SP + 0.1 M potassium chloride + 0.01 M magnesium chloride. The reaction mixture contained 8 nmoles of isopentenyladenosine and 20,000 C.P.M. of [<sup>3</sup>H]-isopentenyladenosine. The 40-60%

and 60-80% ammonium sulfate precipitates were assayed for cytokinin metabolizing activity. The protein concentrations used were 2.3 mg/ml and 2.9 mg/ml, respectively, in total reaction volumes of 0.5 ml. The reactions were run at 37 °C for 24 hours. 0.1ml was removed from each reaction at 0, 0.5, 6, and 24 hours. The reactions were stopped by chilling the aliquots at -20 °C.

In the second run, all assays were run in SP and 0.01 M magnesium chloride. Half of the assays contained 0.1 M potassium chloride as well. The amount of [<sup>3</sup>H]-isopentenyladenosine was raised from 20,000 C.P.M. to 500,000 C.P.M. per assay. The protein content for the second run of the assay was 0.833 mg/ml for the 40-60% proteins and 1.75 mg/ml of the 60-80% proteins. The major difference between this run of the assay and the previous one is that boiled controls were used in this second run. The control proteins were boiled in a water bath for 15 minutes. The reaction volume was increased to 1 ml per assay in the second run. The reaction was run at 30 °C over a period of 48 hours. Two-hundred microliter aliquots were taken at time 0, 8, 24, and 48 hours. All aliquots were immediately chilled at -20 °C.

HPLC was used to isolate and identify the radioactive products of the reaction. Products were eluted from the HPLC column isocratically with 55% methanol. Radioactivity was detected via liquid scintillation counting.

## Detection of Protein in Assay Products

### G-75 Column

Detection of the presence of protein in the reaction product from the first assay was attempted using a Sephadex G-75 gel filtration column. The column used was 40 cm x 1.6 cm, with a volume of 80 ml. The flow rate was set at 10 ml/hr/cm<sup>2</sup>, or 20 ml/hr. The void volume was determined from the elution volume of blue dextran, MW 2,000,000. The 24 hour assay sample with KCl which had protein from the 40-60% ammonium sulfate precipitation was mixed with a solution of protein standards and loaded onto the column. The standards used were the following: 0.3 mg aprotinin (MW 6,500), 0.2 mg cytochrome C (MW 12,500), 0.5 mg carbonic anhydrase (MW 27,000), 0.5 mg albumin (MW 68,000), and 0.8 mg AMP (MW 347). The standards were dissolved in 0.1 ml Tris-HCl buffer (50 mM Tris-HCl with 100 mM KCl pH 7.5) with 5% glycerol.

### Pronase Digestion

The existence of protein in the product of the second assay was determined by the digestion of the product (or lack of it) by pronase. The 30 and 48 hour aliquots were subjected to pronase degradation. The concentration of pronase used was 1 mg/ml, and the reaction was carried out for four hours at 37 °C. HPLC was used to analyze the samples after the pronase treatment. The HPLC elution was carried out isocratically with 55% methanol.

## Results

### In vivo Metabolism of Cytokinins

The results of experiments in which adenine requiring mutants of S. cerevisiae and Sch. pombe were grown in the presence of cytokinins (and the absence of adenine) are summarized in Table 4.

In S. cerevisiae, kinetin was the cytokinin which best supported the growth of adenine auxotrophs. Benzylaminopurine and the cytokinin ribosides supported no visible growth at all. Isopentenyladenine, zeatin, dihydroxyzeatin, and hypoxanthine were intermediate in their ability to promote the growth of adenine-requiring strains.

The Sch. pombe adenine auxotrophs grew very well on all cytokinin supplements provided, including benzylaminopurine and the cytokinin ribosides.

### Synthesis of [<sup>3</sup>H]N<sup>6</sup>- $\Delta^2$ -Isopentenyladenosine

The synthesis of radiolabelled isopentenyladenosine from  $\Delta^2$ -isopentenylbromide and tritiated adenosine was accomplished by the method of Paces et. al. (1971). The success of the synthesis was assessed in two ways: 1) thin-layer chromatography and 2) HPLC.

Before the preparative separation of the radioactive products from the isopentenylation reaction was attempted, a

Table 4.

Growth of adenine auxotrophs of S. cerevisiae on medium supplemented with adenine derivatives.

- + denotes growth similar to that seen in the adenine-containing positive control
- denotes a lack of growth
- +/- denotes intermediate growth

Ade: adenine; Ado: adenosine; i<sup>6</sup>Ade: isopentenyladenine;  
i<sup>6</sup>A: isopentenyladenosine; Z: zeatin; ZR: zeatinriboside;  
DHZ: dihydroxyzeatin; K: kinetin; KR: kinetinriboside;  
BAP: benzylaminopurine; BAPR: benzylaminopurineriboside;  
Hyp: hypoxanthine.

Table 4. Growth of adenine auxotrophs on media supplemented with adenine derivatives

<u>Strain</u>	<u>Supplement</u>											
	Ade	Ado	i <sup>6</sup> Ade	i <sup>6</sup> A	Z	ZR	DHZ	K	KR	BAP	BAPR	Hyp
<u>ade1</u> *	+	+	+	+	+	N.D.	N.D.	+	N.D.	+	+	N.D.
<u>ade2</u>	+	-	+	-	+	-	N.D.	+	-	-	-	+
<u>ade4</u>	+	-	+	-	+	-	N.D.	+	-	-	-	+
<u>ade5</u>	+	-	+	-	+	-	N.D.	+	-	-	-	+
<u>ade6</u>	+	-	+	-	+	-	N.D.	+	-	-	-	+
<u>ade12-2</u>	+	-	+	N.D.	N.D.	N.D.	+	N.D.	N.D.	N.D.	N.D.	-
<u>ade12-40</u>	+	-	+	N.D.	N.D.	N.D.	+	N.D.	N.D.	N.D.	N.D.	-
<u>aah1</u>												
<u>ADE+</u>	+	+	+	+	+	+	+	+	+	+	+	+

\*Sch. pombe

N.D. - Not done

silica gel thin-layer chromatogram was spotted with nonradioactive standard solutions of adenine, adenosine, isopentenyladenine, and isopentenyladenosine and developed as described in Materials and Methods. The R<sub>f</sub> values for the standard solutions were: adenine, 0.49, adenosine, 0.78, isopentenyladenine, 0.70, and isopentenyladenosine, 0.84.

The distribution of the radioactive products from the isopentenylation reaction were determined by recovering the radiolabelled material that comigrated with the ultra-violet absorbing spots of the standards (see Materials and Methods). Radiolabelled compounds were found to comigrate with adenine and isopentenyladenosine. The radioactivity that comigrated with isopentenyladenosine was roughly 63% of the total radioactivity recovered from the chromatogram.

Prior to HPLC, products were initially separated by fractionation on a Sep-Pak cartridge (Waters). Using nonradioactive standards, it was found that adenosine could be eluted with 15% acetonitrile and that 100% acetonitrile eluted the isopentenyladenosine. It was also found that after as little as 5 ml of 15% acetonitrile had gone through the cartridge, isopentenyladenosine began to elute (see Fig. 4). For this reason, the radioactive separation was done using 5 ml of 15% acetonitrile instead of 10 ml. In the radioactive separation, the yield of labelled products was determined by counting 10  $\mu$ l of each 1 ml fraction eluted

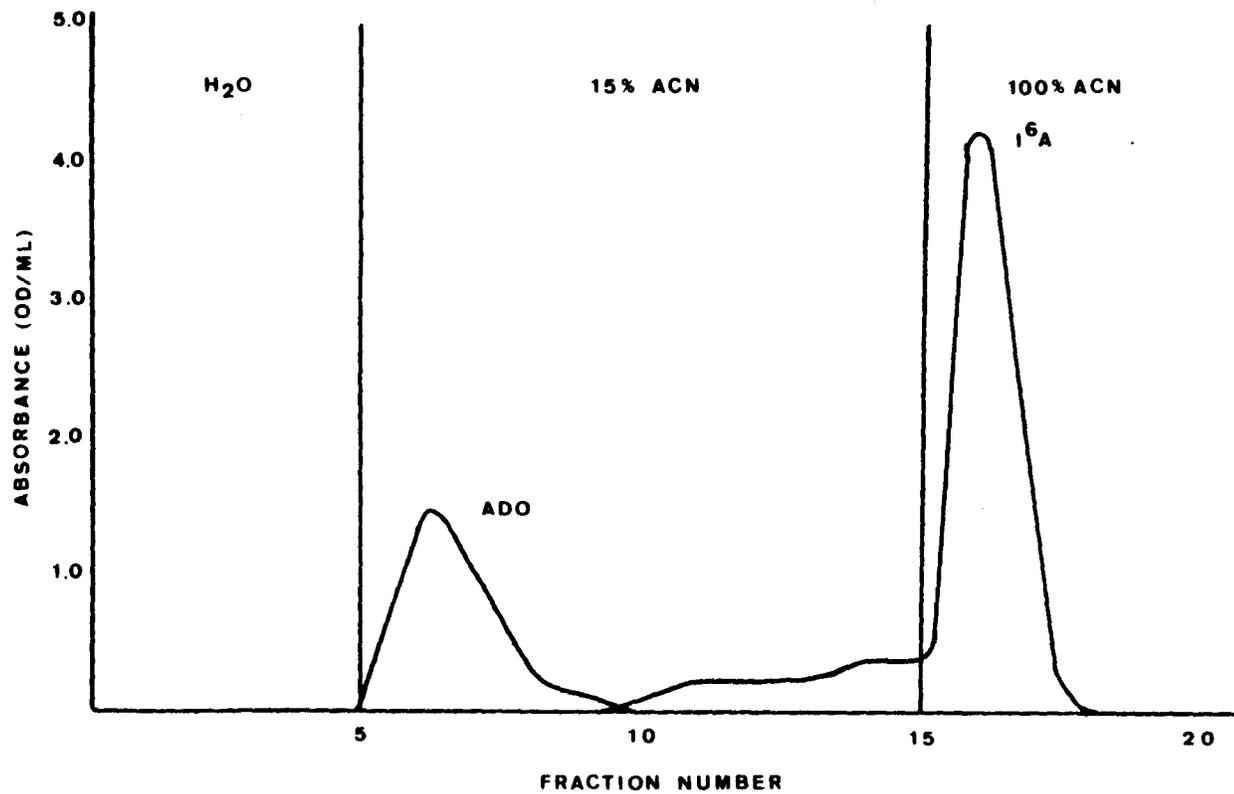
**Figure 4.**

Elution of adenosine and isopentenyladenosine from a Sep-Pak cartridge. Elution was done by a step gradient of 5 ml water followed by 10 ml of 15% acetonitrile and 5 ml of 100% acetonitrile. Fractions were collected to a volume of 1 ml each. Absorbance was measured at 260 nm for adenosine and at 269 nm for isopentenyladenosine.

$i^6A$  = isopentenyladenosine

Ado = adenosine

ACN = acetonitrile



from the cartridge. On the average, the yield of isopentenyadenosine was 60% (see Fig. 5).

In order to verify the purity of the [ $^3\text{H}$ ]-isopentenyladenosine, 10  $\mu\text{l}$  of the first 100% acetonitrile fraction was injected onto an HPLC column along with 10  $\mu\text{l}$  of a 50 OD/ml ( $A_{269}$ ) standard solution of isopentenyadenosine (see Materials and Methods). All of the radioactivity coeluted with isopentenyadenosine.

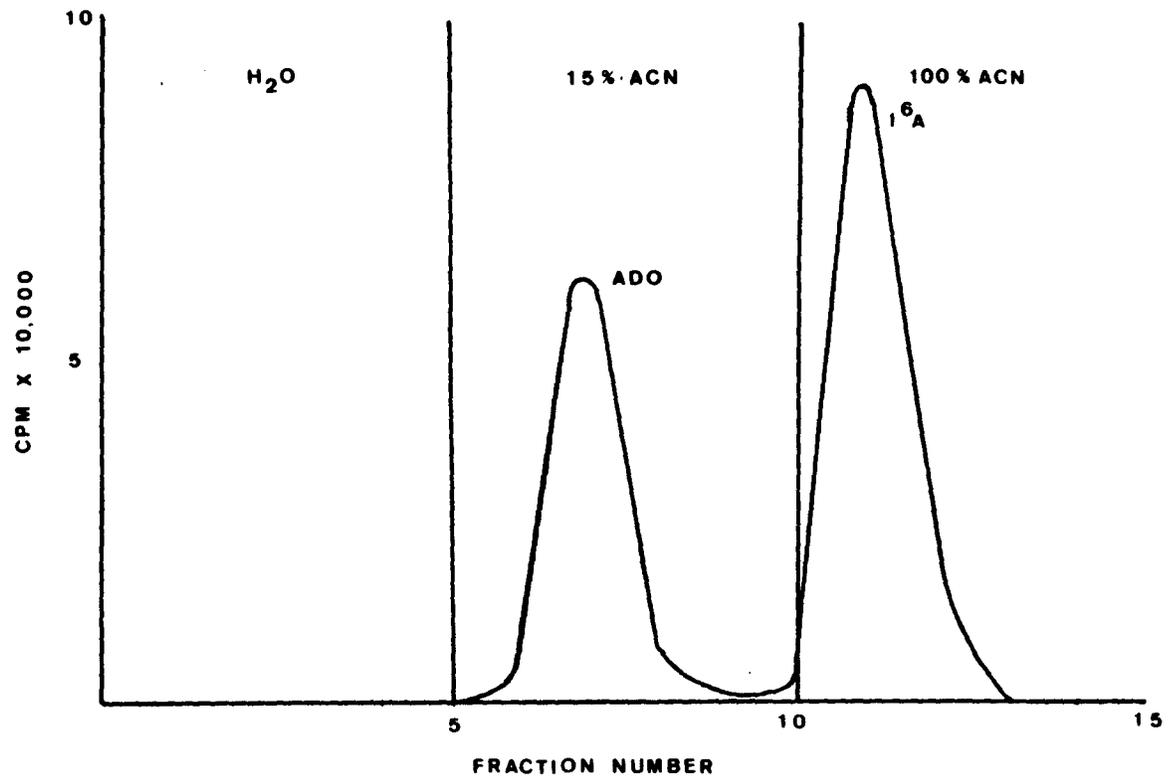
#### Conversion of [ $^3\text{H}$ ]-Isopentenyladenosine to [ $^3\text{H}$ ]-Isopentenyadenine

#### Hydrolysis of [ $^3\text{H}$ ]-Isopentenyladenosine to [ $^3\text{H}$ ]-Isopentenyadenine (1.2 M)

The conversion of [ $^3\text{H}$ ]-isopentenyladenosine to [ $^3\text{H}$ ]-isopentenyadenine was carried out by the hydrolysis described by Martin and Reese (1968). This procedure was attempted with unlabelled isopentenyladenosine, and with the radiolabelled reaction products from the previous synthesis. The nonradioactive hydrolysis products were injected into an HPLC column and the elution profile is shown in Figure 6. From an estimation of the area under the curves, the yield of isopentenyadenine was approximately 7%. A radioactive run using the same procedure yielded the elution profile shown in Figure 7. The amount of isopentenyadenine produced by the hydrolysis reaction was about 2%, based on the

Figure 5.

Elution of radioactive adenosine and isopentenyladenosine from a Sep-Pak cartridge. Elution was done by a step gradient of 5 ml of 15% acetonitrile followed by 5 ml of 100% acetonitrile. Fractions were 1 ml in volume and 0.01 ml was removed from each fraction for counting via liquid scintillation counting.



**Figure 6.**

HPLC elution of nonradioactive products of a 1.2 M isopentenyladenosine HCl hydrolysis. Eluent used was 55% methanol with a flow rate of 0.5 ml/minute. Absorbance was monitored at 254 nm.

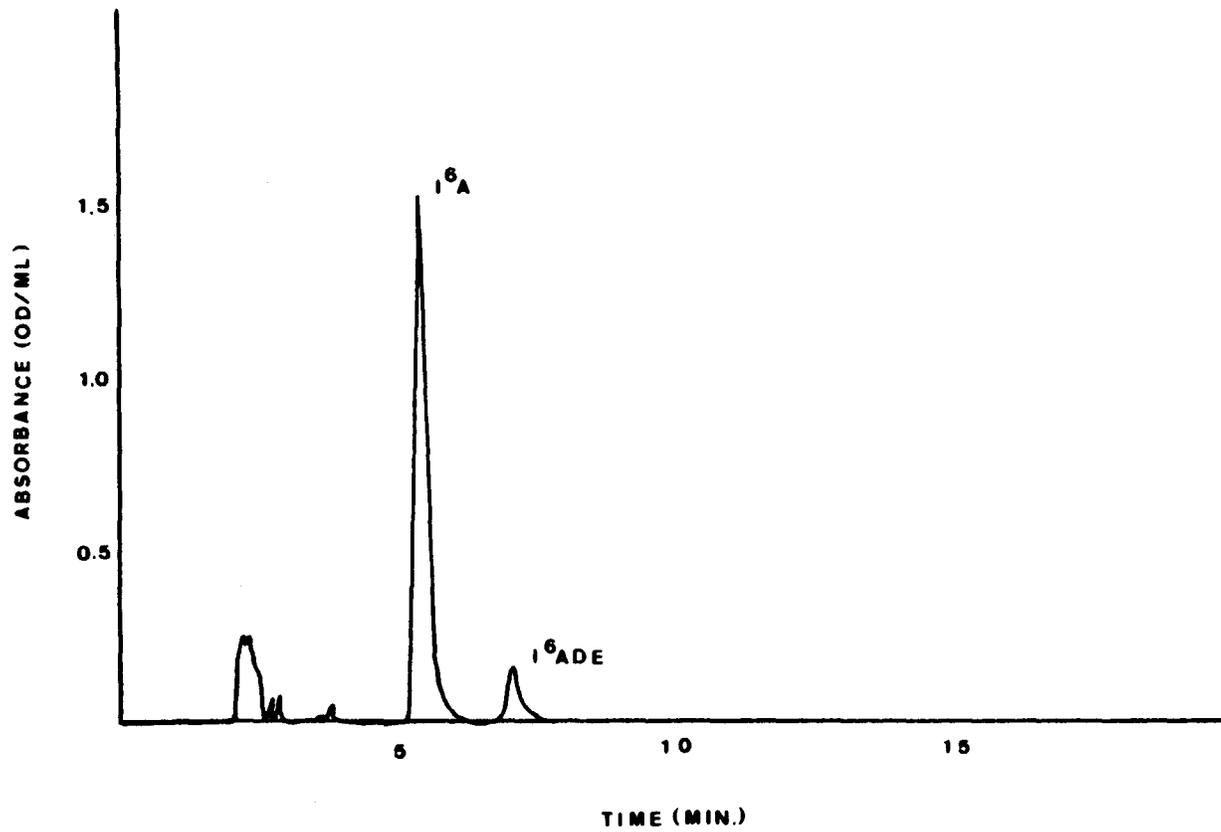
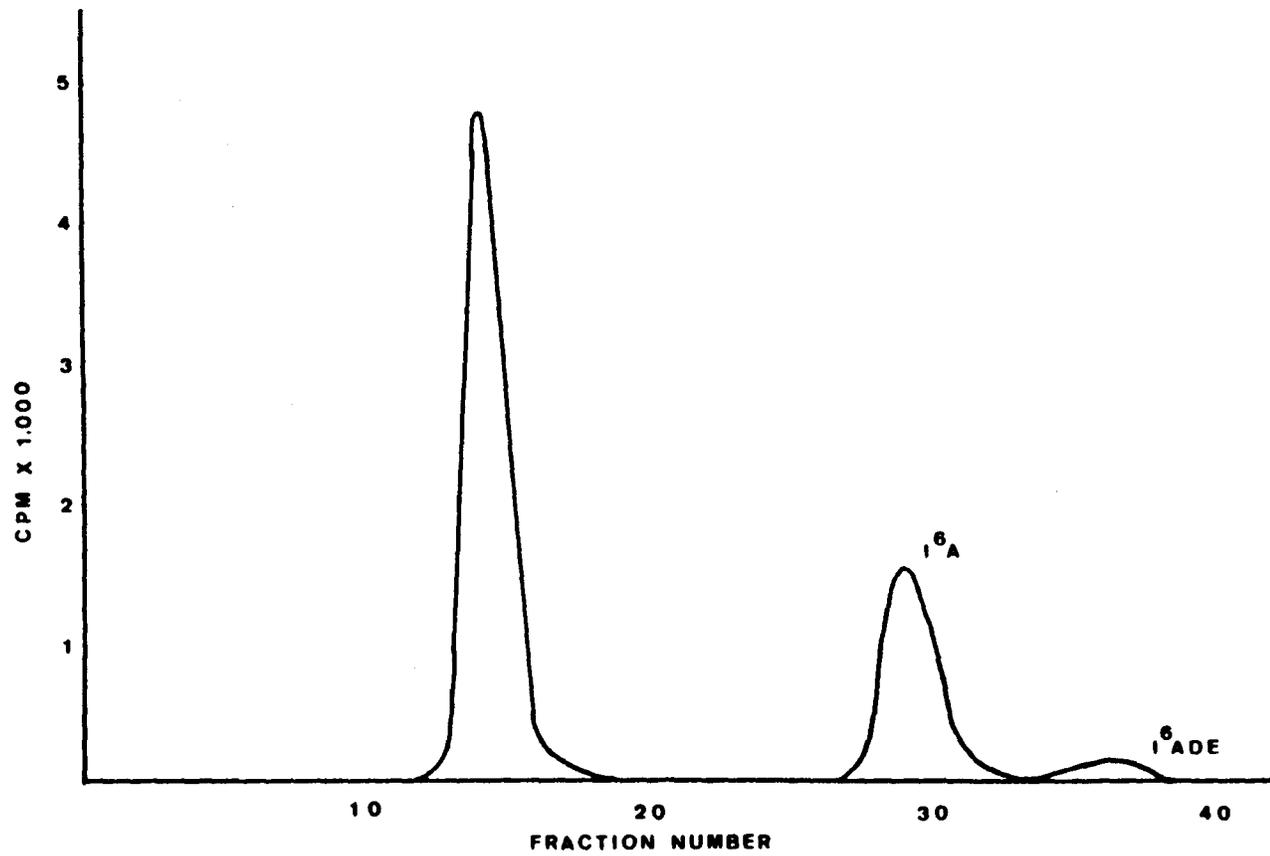


Figure 7.  
HPLC elution profile of elution products of a 1.2 M [<sup>3</sup>H]-isopentenyladenosine HCl hydrolysis. Eluent used was 55% methanol with a flow rate of 0.5 ml/min. Fractions were collected at a rate of 2/min.



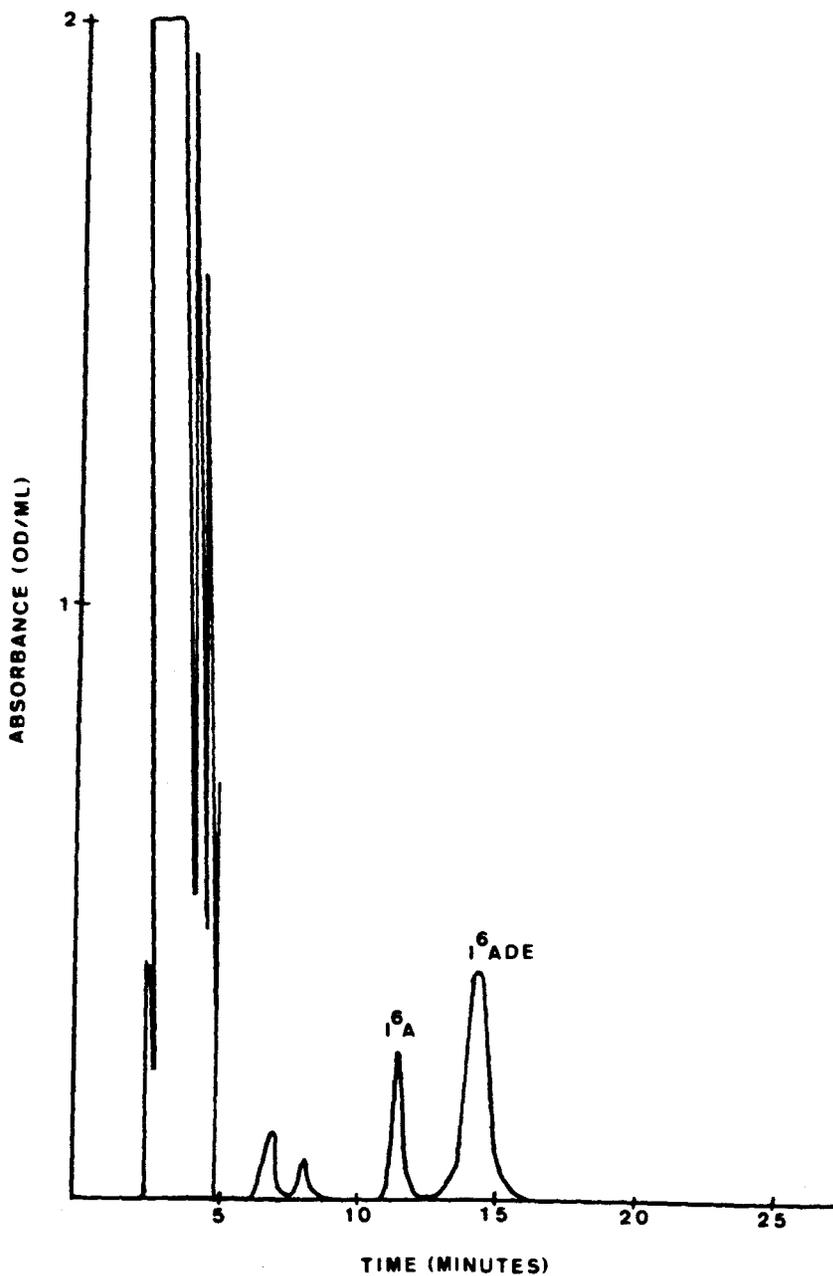
percentage of radioactivity that coeluted from the HPLC column with an isopentenyladenine standard.

Oxidation of [<sup>3</sup>H]-Isopentenyladenosine to [<sup>3</sup>H]-Isopentenyladenine

The conversion of [<sup>3</sup>H]-isopentenyladenosine to [<sup>3</sup>H]-isopentenyladenine by the procedure of Chen *et. al.* (1975) involved the oxidation of the sugar moiety of isopentenyladenosine by sodium periodate followed by the removal of that group. When nonradioactive isopentenyladenosine was subjected to this procedure the yield was about 10%, as can be seen from the HPLC elution profile in Figure 8. In an analogous reaction using the radiolabelled isopentenyladenosine synthesized above, no isopentenyladenine was produced. In fact, in an HPLC run of the reaction products, no isopentenylated compounds were recovered after HPLC fractionation of the oxidation products - including the starting material, [<sup>3</sup>H]-isopentenyladenosine. All of the radioactivity that eluted from the column was found in an early eluting peak of unknown identity (see Fig. 9). To determine the kinetics of isopentenyladenosine breakdown, time points were taken the next time the oxidation of isopentenyladenosine was attempted. One milliliter aliquots were removed from the reaction solution during the boiling procedure at time 0, 10 min, 20 min, and 30 min. The 10 and

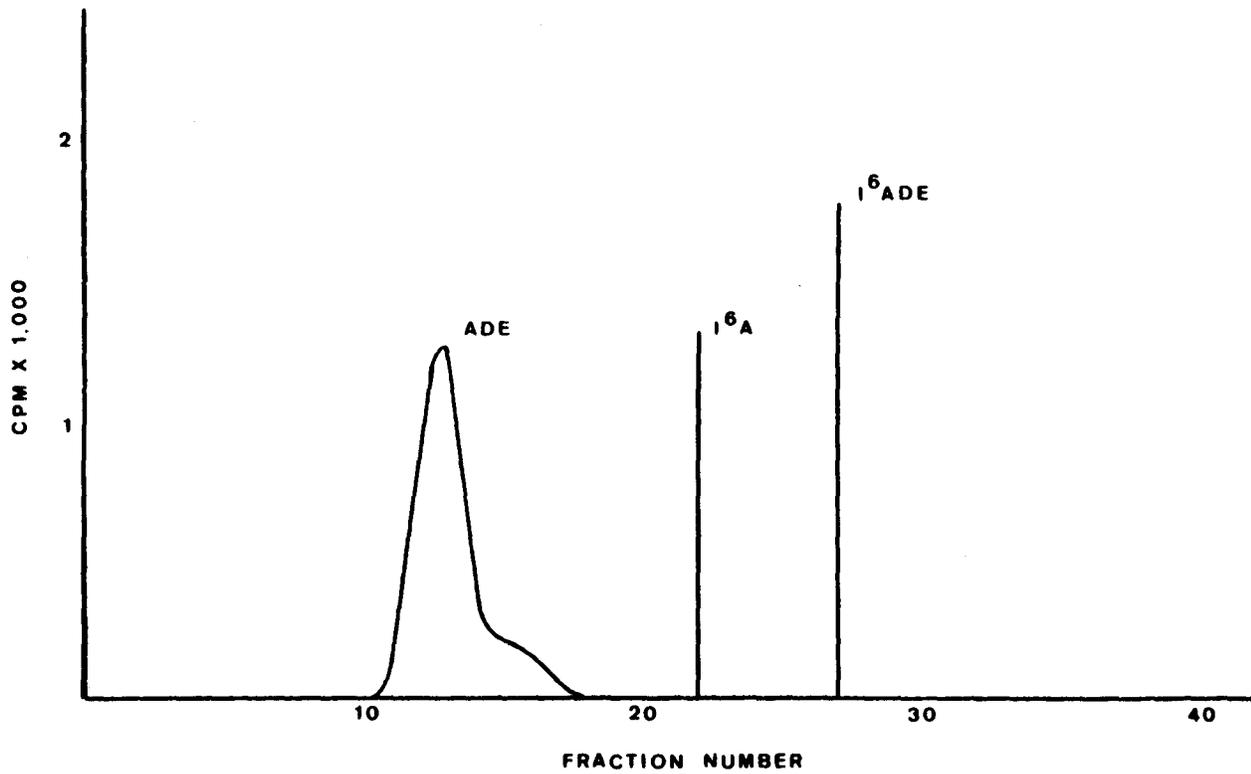
Figure 8.

HPLC profile of nonradioactive products of the oxidation of isopentenyladenosine by sodium periodate. The eluent used was 30% acetonitrile with a flow rate of 0.5 ml/minute. Absorbance was monitored at 254 nm.



**Figure 9.**

HPLC elution of the radioactive products of the oxidation of [<sup>3</sup>H]-isopentenyladenosine by sodium periodate. Eluent used was 30% acetonitrile with a flow rate of 0.5 ml/min. Fractions were collected at a rate of 2 fractions per minute. The i<sup>6</sup>A and i<sup>6</sup>Ade lines indicate when nonradioactive standards of these compounds eluted from the column.



30 minute aliquots were injected onto an HPLC column, and the results were the same as those seen in Figure 9.

Hydrolysis of [<sup>3</sup>H]-Isopentenyladenosine to [<sup>3</sup>H]-Isopentenyladenine (0.015 M)

The hydrolysis of 0.015 M [<sup>3</sup>H]-isopentenyladenosine to [<sup>3</sup>H]-isopentenyladenine by HCl was accomplished using the procedure reported by Martin and Reese (1968). The products formed from the hydrolysis were isopentenyladenine and 3-hydroxy-3-methylbutyl adenine. The isopentenyladenine was identified as such by comigration of the hydrolysis product with an isopentenyladenine standard on a silica gel thin-layer (see Materials and Methods). Isopentenyladenine was also identified by its ultra-violet absorption spectrum. Since a 3-hydroxy-3-methylbutyl adenine standard was not available, its presence in the reaction products could not be verified by thin-layer chromatography. However, the second product found after the hydrolysis of isopentenyladenosine had a peak absorption at the same wavelength as 3-hydroxy-3-methylbutyl adenine (272nm).

The mole fraction of each product and of isopentenyladenosine is plotted in Figure 10. Hydrolysis of radio-labelled isopentenyladenosine was stopped after eight hours. The radioactive reaction mixture was injected onto an HPLC column and a 12% yield of isopentenyladenine was realized, as calculated from the percent of the total

## Figure 10

Mole fractions of isopentenyladenosine and the reaction products, isopentenyladenine and 3-hydroxy-3-methylbutyl adenine, of a 0.015 M isopentenyladenosine HCl hydrolysis over time.

$i^6A$  = isopentenyladenosine

$i^6Ade$  = isopentenyladenine

3H-MBA = 3-hydroxy-3-methylbutyl adenine

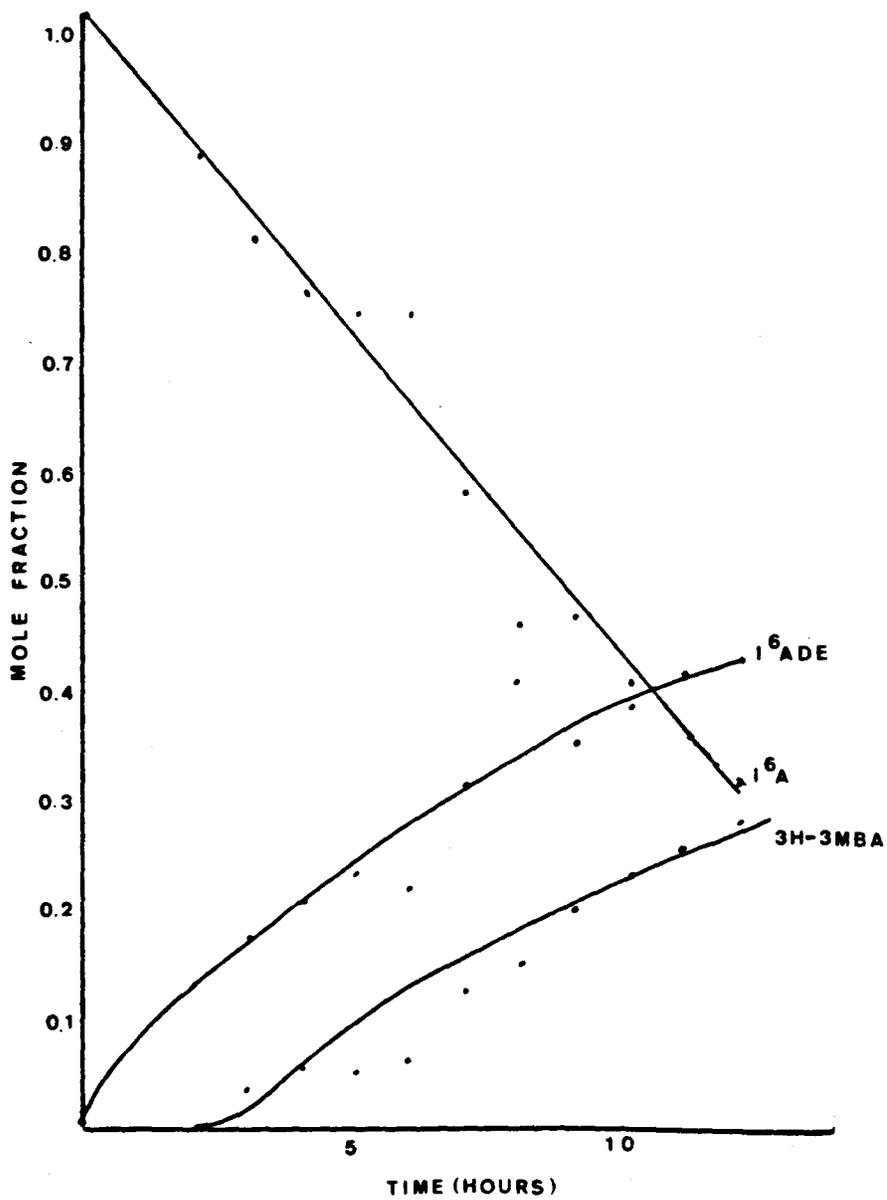
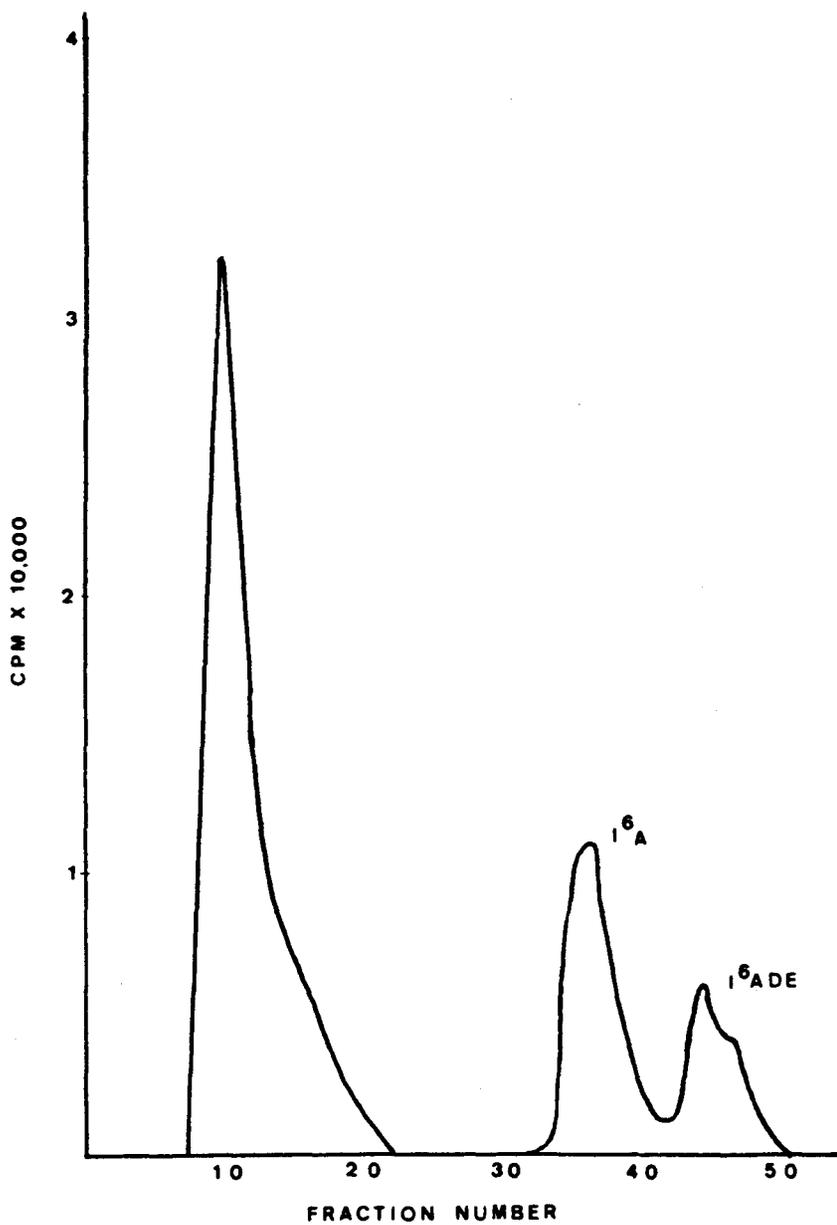


Figure 11.

HPLC elution profile of the radioactive products of a 0.015 M isopentenyladenosine HCl hydrolysis. The eluent used was 30% acetonitrile or 55% methanol, with a flow rate of 0.5 ml/min. Fractions were collected at a rate of 2/min.



radioactivity eluted that comigrated with an isopentenyladenine standard (see Fig. 11).

#### In vivo Uptake and Metabolism of [<sup>3</sup>H]-Isopentenyladenine

The first metabolism studies involved the growth of the *ade-12* and the *aah* strains in liquid SD medium supplemented with [<sup>3</sup>H]-isopentenyladenine (90,500 C.P.M.). The yeast were checked for incorporation of the cytokinin at time points over a period of two days (see Table 3). Surprisingly, no radioactivity was found in either strain at any time point.

To determine if the yeast were able to take up the isopentenyladenine in liquid medium, the *ade-12* strain was grown in SD medium supplemented with 1 mM isopentenyladenine (unlabelled). The *aah-1* strain was not used since its growth in minimal medium is not dependent upon the cytokinin supplement. The *ade-12* strain showed evidence of one doubling after 28 hours of incubation with isopentenyladenine, far fewer divisions than would be expected based on the growth observed on solid medium (see Table 4).

The next experiments utilized solid agar medium, since it was apparent that the cells did not grow well in liquid culture when their only source of adenine is isopentenyladenine. The cells in the solid medium containing [<sup>3</sup>H]-isopentenyladenine (100,000 C.P.M.) did show evidence of growth after four days. However, when the cells were removed

and checked for the presence of radioactivity none was found.

Our next attempt involved the use of yeast carbon base (YCB) as a source of vitamins and minerals instead of yeast nitrogen base (YNB). The reason for this change was that YCB contains no major source of nitrogen while YNB contains ammonium sulfate. The purpose was first to starve the cells for nitrogen by growing them in YCB precultures and then growing them on [<sup>3</sup>H]-isopentenyladenine medium with the isopentenyladenine as the only source of nitrogen. Before this procedure was done using radioactive medium, a nonradioactive liquid culture containing YCB was inoculated with ade-12 cells from a seven day old YCB preculture. Over a period of eight days the cells showed no signs of incorporation. Incorporation was assumed when cell growth occurred. Cell growth was defined as an increase in absorbance of the culture at 625 nm.

In another attempt to enhance the uptake of [<sup>3</sup>H]-isopentenyladenine into the yeast, the antibiotic derivative PBN was included in the radioactive agar medium. PBN has been shown to aid the uptake of hydrophobic compounds such as cytokinins by yeast (Boguslawski, 1985). The optimal concentration of PBN was found to be 0.2 OD/ml ( $A_{260\text{nm}}$ ). Although PBN enhanced the growth of the ade-12 strain on the radioactive medium, there was no effect upon the incorporation of [<sup>3</sup>H]-isopentenyladenine.

### In vitro Metabolism of [<sup>3</sup>H]-Isopentenyladenosine

Although attempts to determine the metabolic products of isopentenyladenine in S. cerevisiae failed because of apparent lack of incorporation, the yeast still must convert isopentenyladenine to adenine to grow on media supplemented with isopentenyladenine. To determine the identity of isopentenyladenine metabolic products, an in vitro study was undertaken in which crude cell extracts were assayed for cytokinin metabolizing enzymes.

The first assay showed the first signs of product formation in the 40-60% ammonium sulfate fraction after 24 hours, in a peak of radioactivity eluting in the early fractions (see Figs. 12-15). This preliminary assay, however, was not accompanied by a control - the early eluting peak observed could have been the result of a chemical breakdown of isopentenyladenosine, rather than the result of enzyme activity. In the second assay, boiled controls were employed. Product formation occurred by 24 hours and increased over time to the end of the reaction at 48 hours. The fact that the controls did not show an increase in product formation suggests that the product formed was due to enzyme activity (see Figs. 16-19).

Possible products formed in the in vitro assay include large molecules, possibly a cytokinin bound to a protein, or a smaller molecule such as an adenine nucleotide or

**Figure 12.**

HPLC elution of the radioactive products of an in vitro assay for cytokinin metabolizing enzymes. Protein assayed in this run were the 60-80% ammonium sulfate precipitated sample. KCl and MgCl were present in the assay mixture. The sample used in this run was a 6 hour aliquot. The eluent used was a step gradient of 14% methanol: 86% 0.01 M  $\text{KH}_2\text{PO}_4$  for 30 minutes, switched to 55% methanol. The flow rate was 1 ml/minute, and the fractions were collected at a rate of 4 fractions per minute for the first 40 minutes, after which time they were switched to 2 fractions per minute.

**Figure 13.**

Same as Figure 12, except that the protein assayed was the 40-60% ammonium sulfate precipitate.

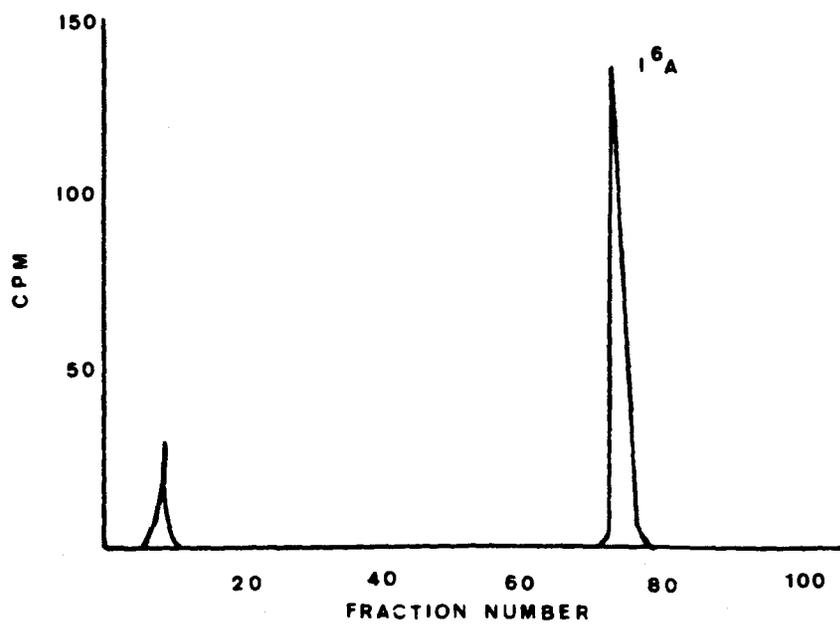
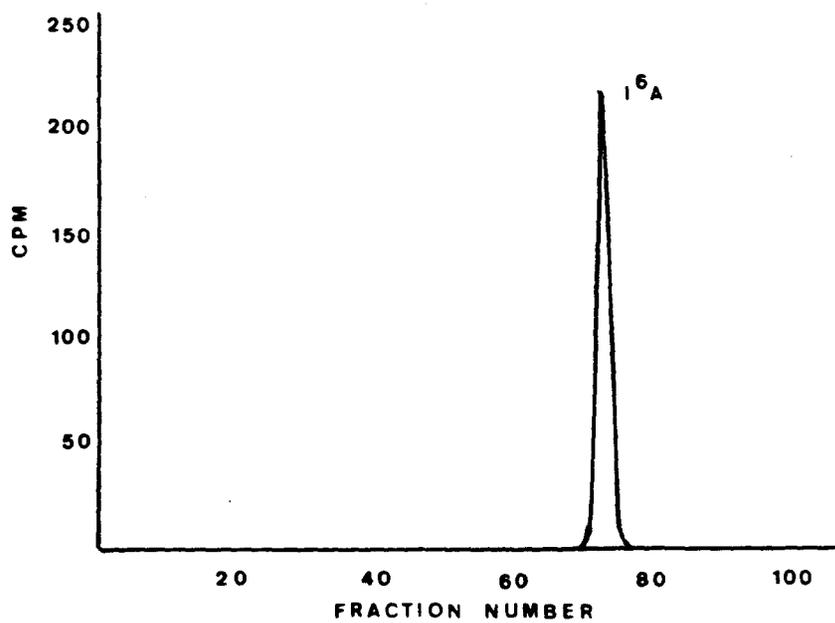


Figure 14.

HPLC elution of the products of the assay involving the 60-80% ammonium sulfate precipitate after 24 hours. Elution conditions were the same as described in Figure 12.

Figure 15.

Same as figure 14, except that the protein involved was the 40-60% fraction.

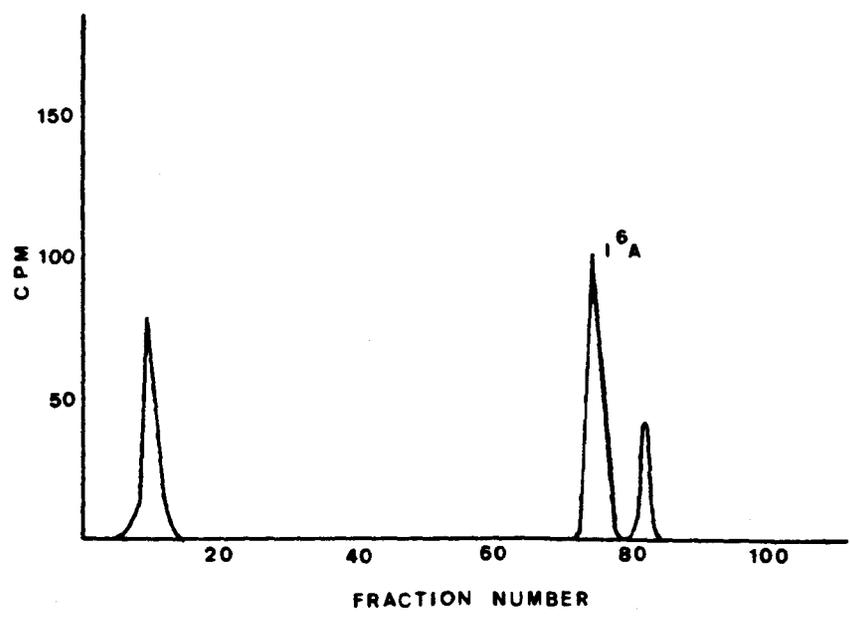
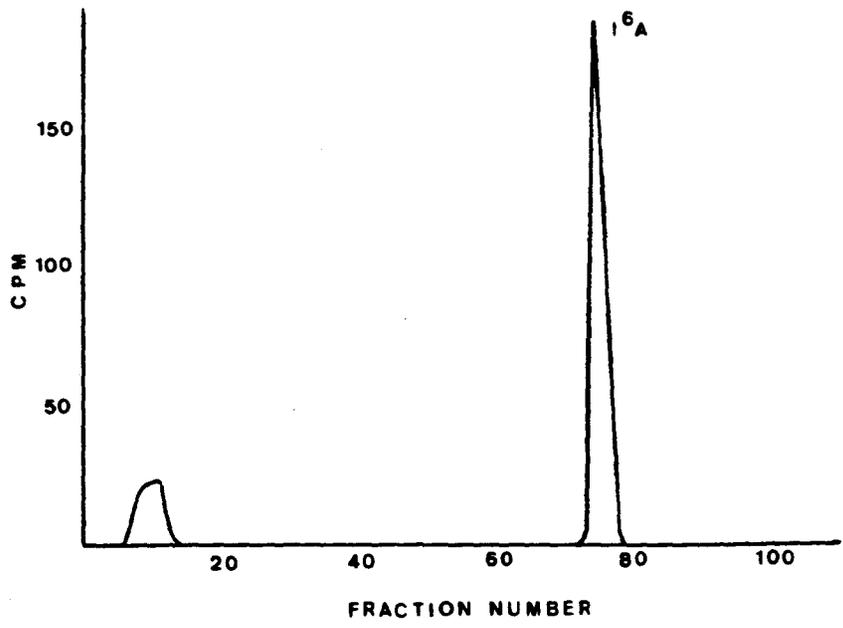


Figure 16.

HPLC elution of the radioactive products of a second in vitro assay for cytokinin metabolizing enzymes. Proteins used in these runs were the 40-60% ammonium sulfate precipitated sample, and a boiled control of the same protein. KCl and MgCl<sub>2</sub> were present in the assay mixture. The samples used in these runs were 8 hour aliquots. The eluent used was 55% methanol. The flow rate used was 0.5 ml/minute and fractions were collected at a rate of 2 fractions per minute. Broken lines indicate the elution of radioactivity in the boiled control samples.

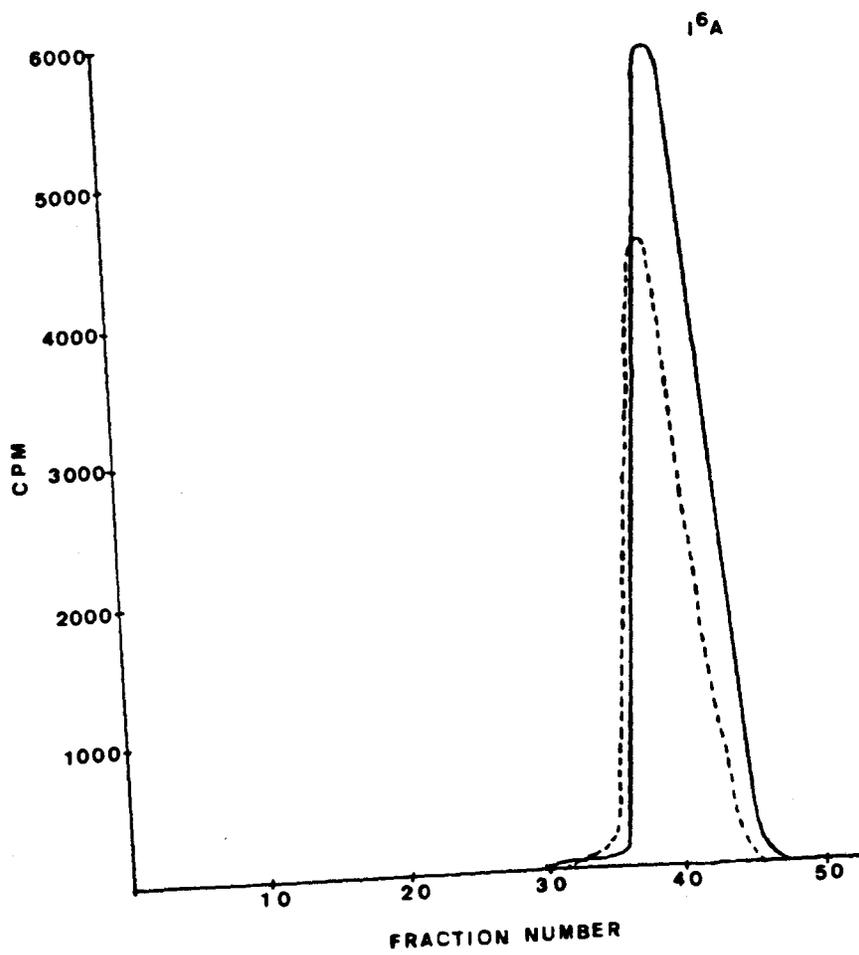


Figure 17.

HPLC elution of the 24 hour products of an assay involving the 40-60% ammonium sulfate precipitated proteins. Eluting conditions were the same as those described in Figure 16.

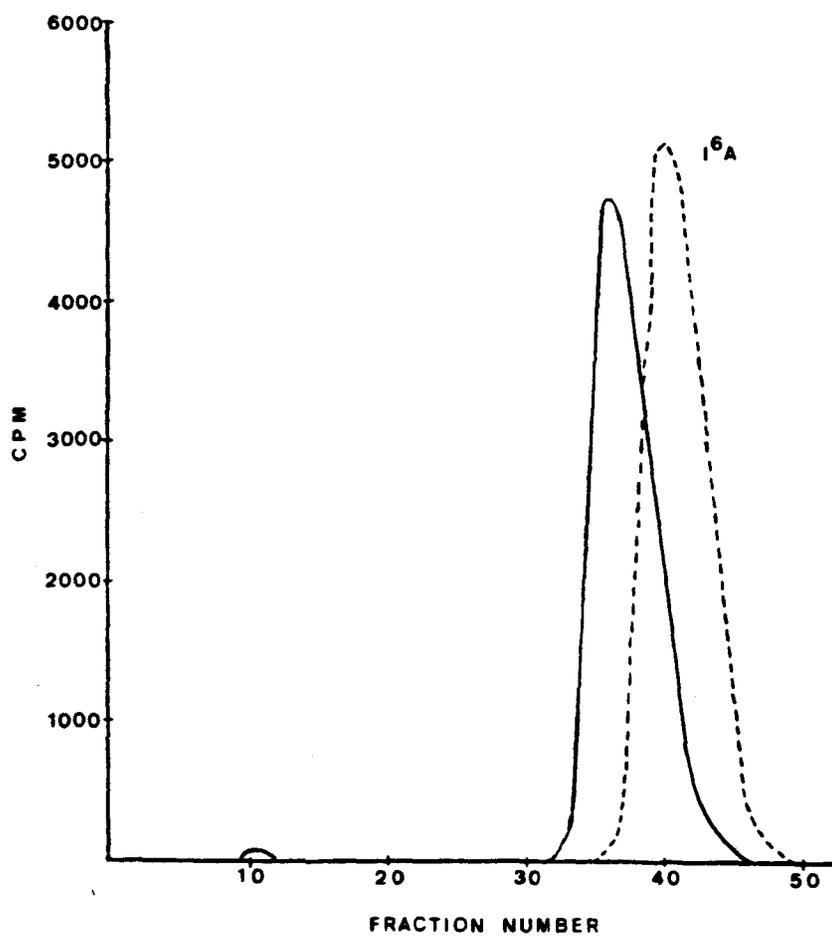


Figure 18.

HPLC elution of a 30 hour aliquot of the same reaction presented in Figures 16 and 17. HPLC elution conditions were the same.

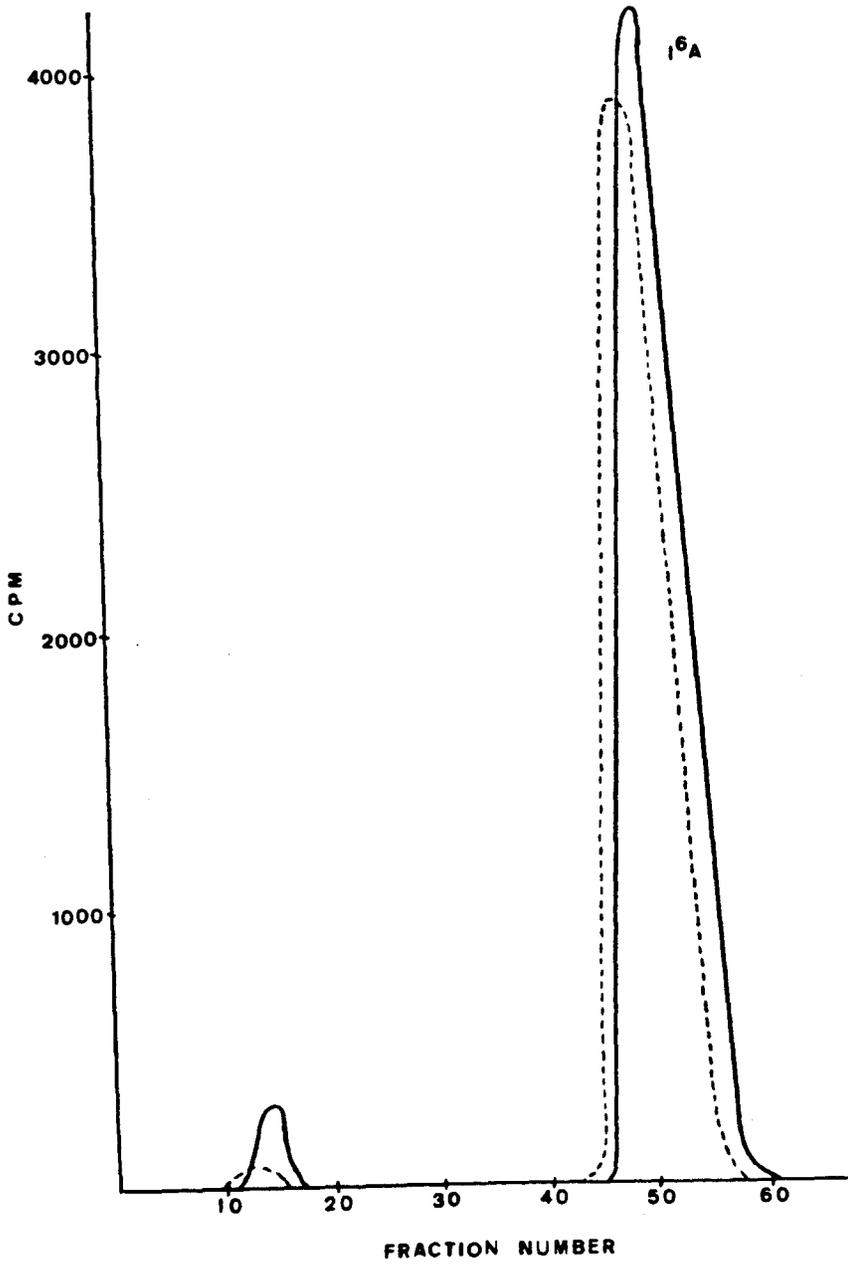
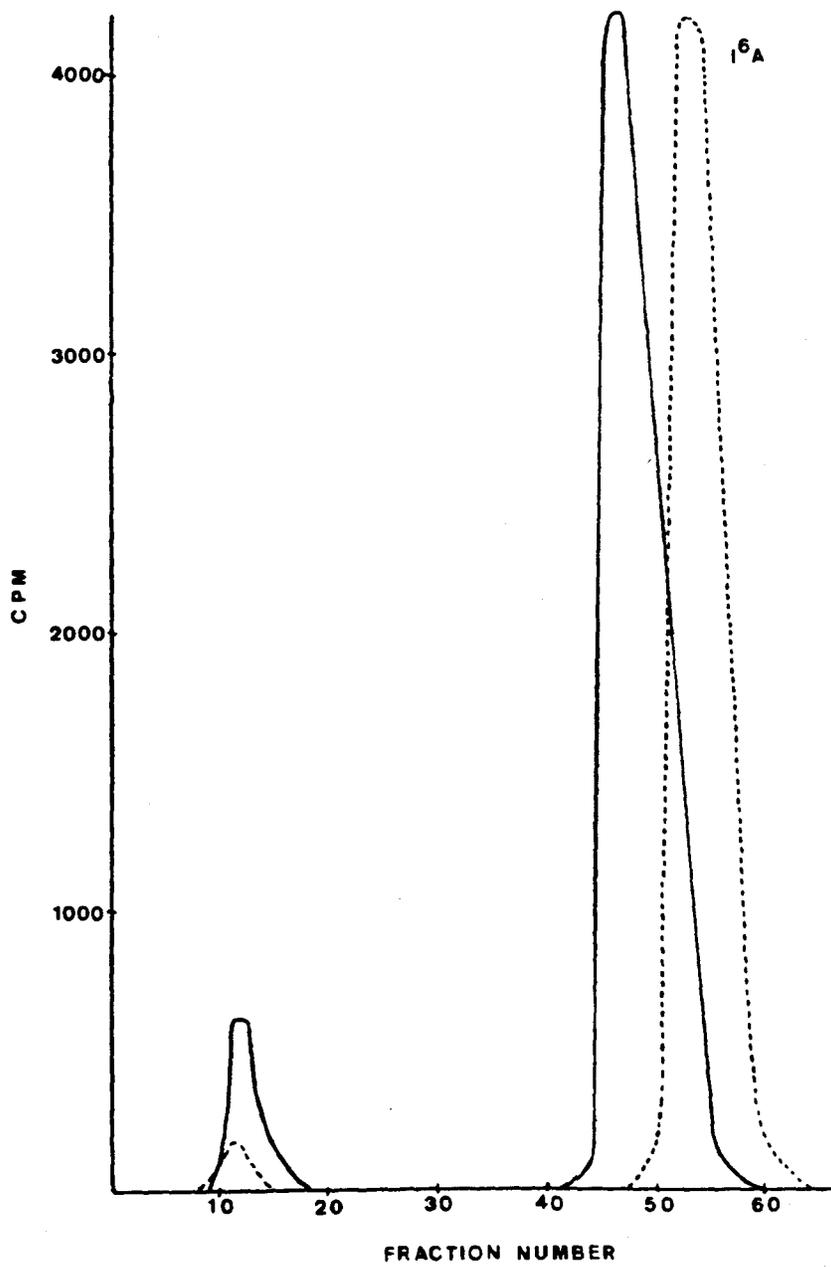


Figure 19.

HPLC elution of a 48 hour aliquot of the assay of 40-60% ammonium sulfate precipitated proteins. HPLC elution conditions were the same as Figures 16-18.

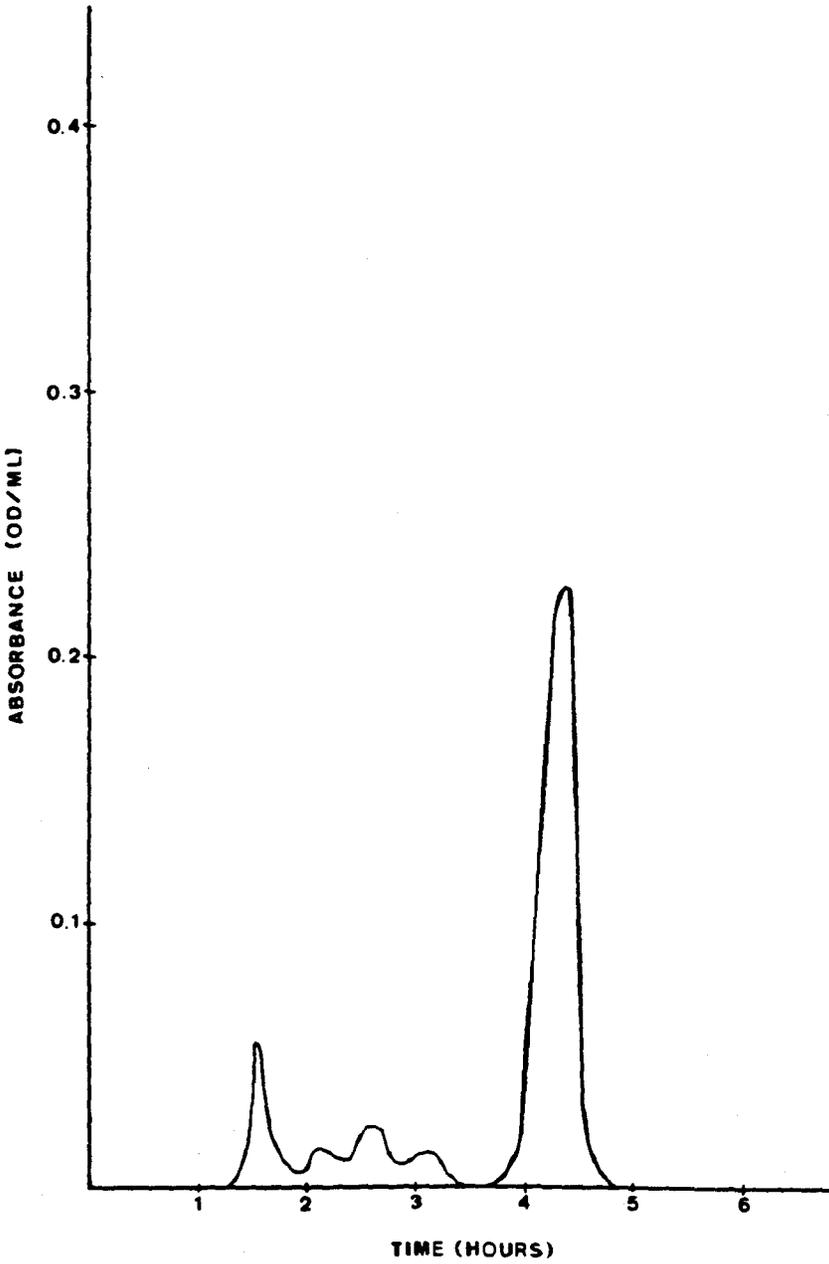


nucleoside. A Sephadex G-75 column was used to determine the size of the radioactive product from the first assay. The elution of the protein standards aprotinin (MW 6,500), cytochrome C (MW 12,500), alcohol dehydrogenase (MW 27,000), albumin (MW 68,000), and AMP (MW 347) is shown in Figure 20. When a sample containing the products of the 24-hour aliquot from the enzyme reaction that assayed the protein from the 40-60% ammonium sulfate fraction (4,000 C.P.M.) was loaded onto the column, no radioactivity was recovered from the column.

Since the radioactive product did not elute from the Sephadex column, its size could not be determined by this method. To determine if the product was protein in nature it was subjected to digestion by pronase. As can be seen from Figure 21, the incubation of the reaction products with pronase did not alter the appearance of the early eluting peak presumed to be the reaction product. This seems to indicate that the radioactive product was not a protein.

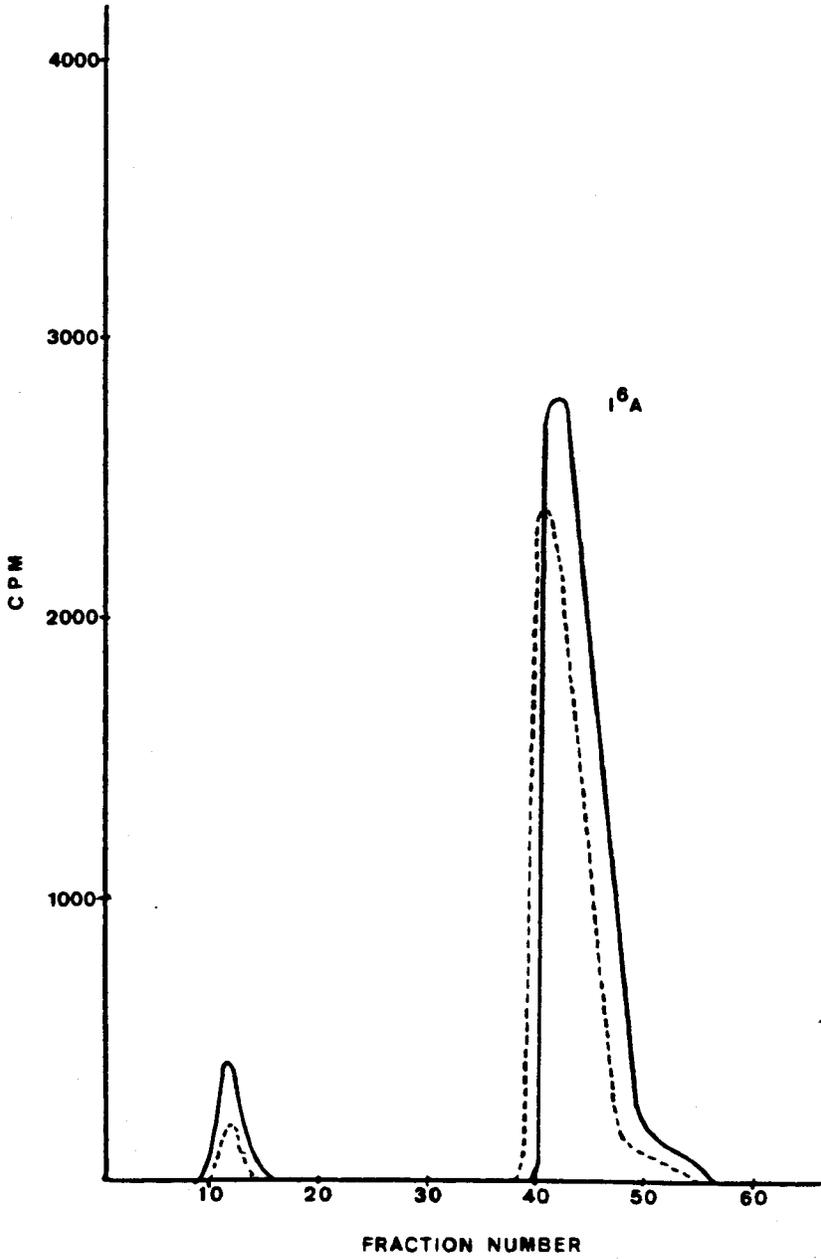
Figure 20.

Elution profile of protein and AMP standards from a Sephadex G-75 column. The eluent used was 50 mM Tris-HCL with 100 mM KCl (pH 7.5). The flow rate was 20 ml/hour and the absorbance was monitored at 280 nm. The protein standards used in this elution were aprotinin (MW 6,500), cytochrome C (MW 12,500), alcohol dehydrogenase (MW 27,000), albumin (MW 68,000). AMP has a molecular weight of 347.



**Figure 21.**

HPLC elution profile of the reaction products from the in vitro assay after pronase treatment. Sample presented here was the 48 hour aliquot. The eluent used was 55% methanol. The flow rate used was 0.5 ml/min, and fractions were collected at the rate of 2/min.



## DISCUSSION

To date, the only studies performed on the metabolism of N<sup>6</sup>-substituted purines in yeast are those done by Abbondandolo et. al. (1971), and Guern et. al. (1972). Both of these studies were done in Schizosaccharomyces pombe. Abbondandolo et. al. (1971) found that Sch. pombe converted N<sup>6</sup>-substituted purines, such as 6-chloropurine to hypoxanthine by the action of adenine aminohydrolyase (AAH). The hypoxanthine is then converted to IMP by hypoxanthine guanine phosphoribosyltransferase, and the IMP is converted to AMP by the last two enzymes in the AMP biosynthetic pathway (see Fig. 3). Guern et. al. (1972) found that cytokinins are metabolized by the same metabolic pathway in Sch. pombe.

The present study provides in vivo and in vitro evidence that the yeast Saccharomyces cerevisiae uses an alternative method for the metabolism of cytokinins. S. cerevisiae may possess a cytokinin oxidase-like enzyme similar to that found in Zea mays (McGaw and Horgan, 1983). In the in vivo metabolism study it first became apparent that S. cerevisiae must have a mechanism for cytokinin breakdown independent of that seen in Sch. pombe. The presence of enzyme(s) which form a product from

isopentenyladenosine in vitro also suggests an alternative metabolism system because although its identity is unknown, the product is not hypoxanthine nor one of its derivatives.

Since the studies of Abbondandolo et. al. (1971) and Guern et. al. (1972) showed that the N<sup>6</sup>-substituted purines they used were metabolized to AMP by Sch. pombe, we hypothesized that cytokinins given to S. cerevisiae would be similarly converted to AMP. This was tested by growing various mutant strains of S. cerevisiae and one of Sch. pombe, which required adenine for growth, on media supplemented with various cytokinins and their ribosides in place of adenine. We found that Sch. pombe and S. cerevisiae were able to grow on most of the cytokinin supplements provided (Table 4). From these observations we concluded that S. cerevisiae as well as Sch. pombe has the ability to convert cytokinins to AMP. Of interest is the fact that S. cerevisiae did not metabolize any of the cytokinin ribosides. This may be due to a problem in the transport of the ribosides into the yeast cell. It was shown by Demain (1964) that adenine requiring strains of S. cerevisiae can not grow when adenosine is substituted for adenine. His results suggested that adenosine can not penetrate the yeast cell and/or membrane.

The observation in this study which led us to conclude that S. cerevisiae has a different cytokinin-metabolizing pathway was that the ade-12 strains were able to grow when

cytokinins were substituted for the normal adenine requirement. The mutation in these strains is in a gene coding for an enzyme necessary for the conversion of IMP to AMP, adenylyl succinate synthetase (Lomax and Woods, 1970). If the only route for cytokinin metabolism is via adenine aminohydrolase, this strain should not be able to synthesize AMP since it can no longer form AMP from IMP. The fact that it grew indicates another way to synthesize AMP. The N<sup>6</sup> side chain may be removed with a cytokinin oxidase-like enzyme, thus forming adenine. The adenine may then be converted to AMP via adenine phosphoribosyltransferase. An alternative to this is the possibility of formation of cytokinin monophosphates via adenine phosphoribosyltransferase, and the subsequent removal of the N<sup>6</sup> side chain leaving AMP. Both of these routes have been found to be present in higher plants (Letham, 1978).

The synthesis of [<sup>3</sup>H]-isopentenyladenosine by the method of Paces et. al. (1971) was relatively successful, and about a 60% yield was achieved. The conversion of [<sup>3</sup>H]-isopentenyladenosine to [<sup>3</sup>H]-isopentenyladenine, however, proved more challenging. The method which produced the highest yield was the hydrolysis of a 0.015 M solution of [<sup>3</sup>H]-isopentenyladenosine in HCl, with a 12% yield.

The hydrolysis of a 1.2 M solution of [<sup>3</sup>H]-isopentenyladenosine in HCl at high temperature seemed to result in the removal of the N<sup>6</sup> side chain in addition to the ribose. The

same phenomenon was observed when sodium periodate was used to oxidize isopentenyladenosine to isopentenyladenine. It seems that the concentration of isopentenyladenosine is critical in both procedures. The fact that the first hydrolysis reaction was done on a very small scale may account for the low yield of this method. When a reaction volume of 50  $\mu$ l was used small errors in concentration became greatly magnified. The procedure as reported by Martin and Reese (1968) did not utilize isotopes and the volumes were much larger which minimized errors. We required small volumes in order to maintain the highest specific activity possible.

Although the oxidation procedure of Chen et. al. (1975) was designed for use with radioactive isopentenyladenosine, we again encountered concentration problems. At the time we attempted the procedure we had not yet purified the solution containing the products of the isopentenylation reaction. The presence of 40% adenosine was apparently enough to cause the reaction to fail (D. Crumrine, personal communication). We are confident that this was the problem since we obtained the same results when the oxidation was run using a nonradioactive mixture of 40% adenosine and 60% isopentenyladenosine as the reaction substrate. By the time we had purified the [<sup>3</sup>H]-isopentenyladenosine from the isopentenylation reaction product solution, we had tried a nonradioactive run of the 0.015 M isopentenyladenosine HCl

hydrolysis, and found that yield was preferable to that obtained from the periodate procedure. The yield of the radioactive run of this hydrolysis, also devised by Martin and Reese (1968), was as stated above, about 12%.

All attempts to demonstrate incorporation of [<sup>3</sup>H]-isopentenyladenine into S. cerevisiae cells were unsuccessful. The first attempt was done in radioactive liquid medium, and no radioactivity was present in the cells after two days. Since the previous in vivo studies with cytokinins were done on solid agar medium we looked for incorporation of the [<sup>3</sup>H]-isopentenyladenine by growing the yeast on solid agar medium. Growth was observed in the same manner as in the previous study, but the cells contained no radioactivity.

YCB was used in the precultures and growth cultures in the next experiments in an attempt to starve the cells for nitrogen and then supply nitrogen in the form of isopentenyladenine. The incorporation of isopentenyladenine did not seem to be enhanced by this procedure which utilized unlabelled isopentenyladenine. For this reason the YCB experiments were never attempted using radioactive isopentenyladenosine.

In the last set of incorporation experiments the antibiotic derivative PBN was used. PBN has been shown to enhance the uptake of hydrophobic compounds such as cytokinins by yeast (Boguslawski, 1985). We also tested this

assumption with unlabelled cytokinins before attempting the radioactive incorporation with the PBN. We found that a concentration of 0.2  $A_{260}$ /ml, was optimal for isopentenyladenine uptake and growth in the ade-12 cells. Although PBN appeared to increase the amount of isopentenyladenine taken into the cells (the growth was enhanced), the cells were still found to contain no radioactivity.

A possible reason for the lack of intracellular radioactivity in these experiments could be the low specific activity of the [ $^3$ H]-isopentenyladenine in the medium. To see growth on medium in which isopentenyladenine has replaced adenine a concentration of 1 mM isopentenyladenine must be supplied. The addition of such a large quantity of nonradioactive isopentenyladenine may have lowered the specific activity of the compound below detectable limits. During the isopentenylation reaction the specific activity of the tritiated adenosine was reduced by a factor of about 1360 by the addition of unlabelled adenosine. The addition of unlabelled isopentenyladenine to the incorporation media reduced the specific activity by an additional factor of 85. For every labelled molecule of isopentenyladenine in the media there are 115,600 unlabelled molecules.

The in vitro enzyme assay performed suggested that S. cerevisiae can metabolize isopentenyladenosine. It should be noted here that [ $^3$ H]-isopentenyladenosine was used instead of [ $^3$ H]-isopentenyladenine. The problem with the [ $^3$ H]-

isopentenyladenosine metabolism in vivo appears to be the transport of the riboside into the yeast cell (Demain, 1964). This problem was eliminated in the in vitro study. In addition, we were able to synthesize the ribose form of [<sup>3</sup>H]-isopentenyladenine much more easily than the base (see Results).

The assay used was derived from a procedure devised by McGaw and Horgan (1983) to detect the presence of cytokinin oxidase in Zea mays. As can be seen from figures 14-19, isopentenyladenosine was converted to a radioactive product. The identity of the product remains unknown. It does not migrate with hypoxanthine or its derivatives in the HPLC system. The presence of a product other than hypoxanthine or its derivatives argues in favor of an alternate pathway for cytokinin metabolism in S. cerevisiae.

In light of the evidence collected in our lab it seems that in yeast isopentenyladenosine may be bound to protein (H. Laten, personal communication). To determine if our radiolabelled product was bound to a protein, the reaction product solution was loaded onto a Sephadex G-75 column along with several protein standards. The elution of these standards is shown in Figure 24. The radioactivity loaded on the column was never retrieved - the product appeared to bind to Sephadex. Gel-solute interactions involving cyclic aromatic compounds such as purines and pyrimidines have been previously reported (Janson, 1967). We next used a pronase

digestion to assess the presence of proteins in the reaction product. The HPLC elution of the product was unchanged by incubation with 1 mg/ml of pronase, indicating the absence of protein in the product (see Figs. 21 and 22). As of this time, the identity of the product is unknown.

Possible products of the in vitro metabolism of isopentenyladenosine include adenine, adenosine, AMP, ADP, ATP, isopentenyladenine, isopentenyladenosine monophosphate, hypoxanthine, inosine, and IMP. If the enzyme activity seen in the in vitro assay is that of a cytokinin oxidase-like enzyme, the N<sup>6</sup> side chain would be removed and adenosine would result. If a nucleosidase was present as well in the crude extract assayed, adenine could be formed. If only the nucleosidase was present, isopentenyladenine would be formed. If the metabolism of isopentenyladenosine in S. cerevisiae is carried out by adenosine aminohydrolase as in the studies by Hall et. al. (1971) and McLennan et. al. (1968), inosine and perhaps hypoxanthine would result. The activity of kinases and phosphoribosyltransferases in the assay mixture could produce AMP, IMP, and isopentenyladenosine monophosphate.

HPLC has eliminated all of the possible in vitro metabolic products mentioned above. All of the bases and ribosides have longer retention times on the HPLC column than the radioactive product with the solvent system used. The remaining possibilities are the adenine nucleotides and

IMP. Although the radioactive metabolite eluted at about the same time as these compounds it could not be said to co-elute with any of them.

It is possible that yeast contain other metabolic enzymes which may convert isopentenyladenosine to other, as yet undetected derivatives. Chen (1982) described mixed function oxidases which hydroxylate isopentenyladenine and isopentenyladenosine to zeatin and zeatin riboside, respectively. Perhaps there are enzymes such as this in yeast which can catalyze the transformation of isopentenyladenosine to other cytokinins or other compounds not before seen in this organism.

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**APPENDIX A**

## APPENDIX A

### Incorporation of [<sup>14</sup>C]-Adenine Into Selected Strains of *S. cerevisiae*

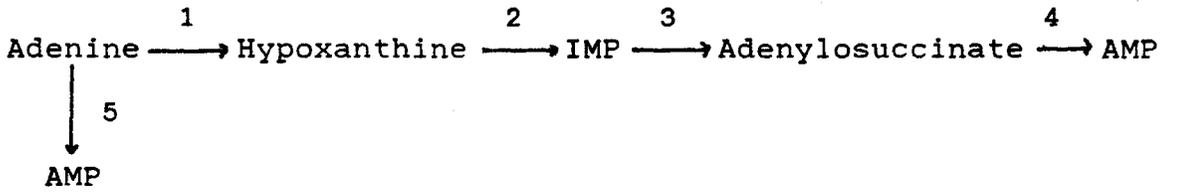
In attempting to establish the presence of a cytokinin oxidase-like activity in *S. cerevisiae*, it was desirable to eliminate the known alternative mechanism of cytokinin metabolism involving adenine aminohydrolase. A strain of *S. cerevisiae* lacking adenine aminohydrolase (aah-1) was kindly provided by Dr. R.A. Woods. We also received an adenine phosphoribosyltransferase mutant (apt-1), and a double mutant lacking both enzymes.

Before using any of these strains we attempted to check their phenotypes via adenine labelling. We planned to grow the mutants in the presence of radioactive adenine, break open the cells and analyze the radioactive products. We expected that the aah-1 mutant would produce no radioactive hypoxanthine or IMP. The double mutant should have produced no adenine metabolites at all, no AMP, IMP, hypoxanthine, DNA or RNA. The apt-1 mutant would still produce all of these products since it is not lacking the adenine aminohydrolase enzyme or the hypoxanthine guanine phosphoribosyltransferase enzyme which converts hypoxanthine to IMP, an intermediate in the AMP biosynthetic pathway (see Fig. 22).

Figure 22.

The metabolism of adenine in S. cerevisiae

1. Adenine aminohydrolase,
2. Hypoxanthine guanine phosphoribosyltransferase,
3. Adenylosuccinatesynthetase,
4. Adenylosuccinate lyase,
5. Adenine phosphoribosyltransferase.



### Labelling Procedure

The precultures used were 50 ml YPD liquid medium. They were inoculated with strains 37 (+), 1520 (apt-1), 1530 (apt-1 aah-1), and 1540 (aah-1). They were grown overnight in a 30 °C shaker at 300 rpm. Growth cultures contained 50 ml of SD medium to which 10  $\mu$ Ci [ $^{14}$ C]-adenine was added. 0.5 ml of each preculture was added to one of the four growth culture flasks. The radioactive cultures were grown at 30 °C in a 300 rpm shaker. Eight ml aliquots were taken at 0, 5, 15, 30, and 240 minutes. Cells were harvested by centrifugation at 15,000 G for 15 minutes.

### Cell Lysis Procedure

The cells were mechanically broken in a 1.5 ml microfuge tube containing 0.1-0.2 ml lysis buffer (0.05 M Tris, 0.01 M MgCl<sub>2</sub>, 0.1 mM EDTA, 1% SDS pH 7.5) and 0.1-0.2 ml acid washed glass beads. The tube was vortexed on high for 1 minute and cooled on ice for 30 seconds. This was repeated a minimum of five times. The tube was centrifuged for 30-60 seconds in an Eppendorf microcentrifuge. The supernatant was decanted and 0.5 ml of lysis buffer was added to the debris and beads. This was recentrifuged and the two supernatants combined and checked for radioactivity by counting a small aliquot in a liquid scintillation system.

### Analysis of Cell Contents

The supernatants from the lysed cells were filtered and injected onto an HPLC column for analysis. The samples which were so analyzed were the four hour samples from all four strains and the 15 minute sample from strain 1520. The solvent system used to elute all samples from the HPLC column was a step gradient of 14% methanol : 86% 0.1M  $\text{NaH}_2\text{PO}_4$  (v/v) followed after 30 minutes by 55% methanol.

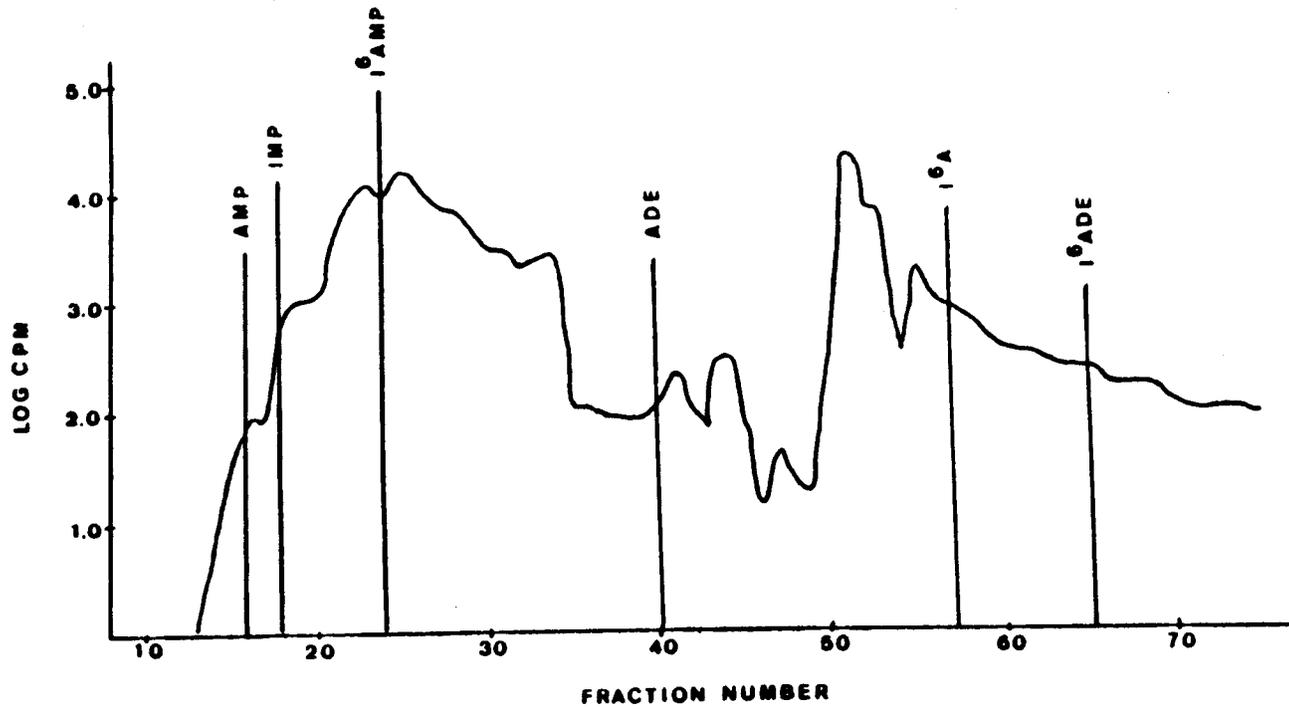
Our results show many early eluting, broad peaks. Figure 23 is representative of what we found in all five samples. We suspected that they consisted of DNA and RNA. It was later shown by Laten and Valentine that subjecting the supernatants to trichloroacetic acid (TCA) precipitation prior to HPLC analysis removed the early eluting peaks.

Although the DNA and RNA could be removed by TCA precipitation, according to our original hypothesis they should not have been present at all in strain 1530. Furthermore, HPLC analyses of the TCA precipitated samples revealed radioactive hypoxanthine, inosine, and most unexpectedly adenosine in all of the samples. Until this time no enzyme capable of ribosylating adenine had yet been found in yeast.

This apparent phosphorylase enzyme opens up a number of possible pathways for adenine metabolism. If the yeast forms adenosine and then AMP it has bypassed the apt enzyme. Also,

Figure 23.

HPLC elution profile of the metabolic products of adenine in S. cerevisiae. The eluent used was a step gradient of 14% methanol : 86% 0.01M  $\text{KH}_2\text{PO}_4$  for 30 minutes, followed by 55% methanol. The flow rate was 0.5ml/min and fractions were collected at the rate of 4 per minute, switched to one every 2 minutes after the first 10 minutes.



they can form inosine and IMP by forming adenosine and being deaminated by adenosine aminohydrolyase, making the adenine aminohydrolyase enzyme unnecessary (see Fig. 24).

These results, although interesting, did not accomplish the phenotype check the adenine label was intended for. We requested the procedures used to isolate the mutants from Dr. Woods to check the strains for their mutations. Since the aah-1 strain (1540) would be used in the isopentenyl-adenine metabolism studies, its phenotype was verified using Woods' method (Woods et. al., 1984).

#### Detection of AAH Activity

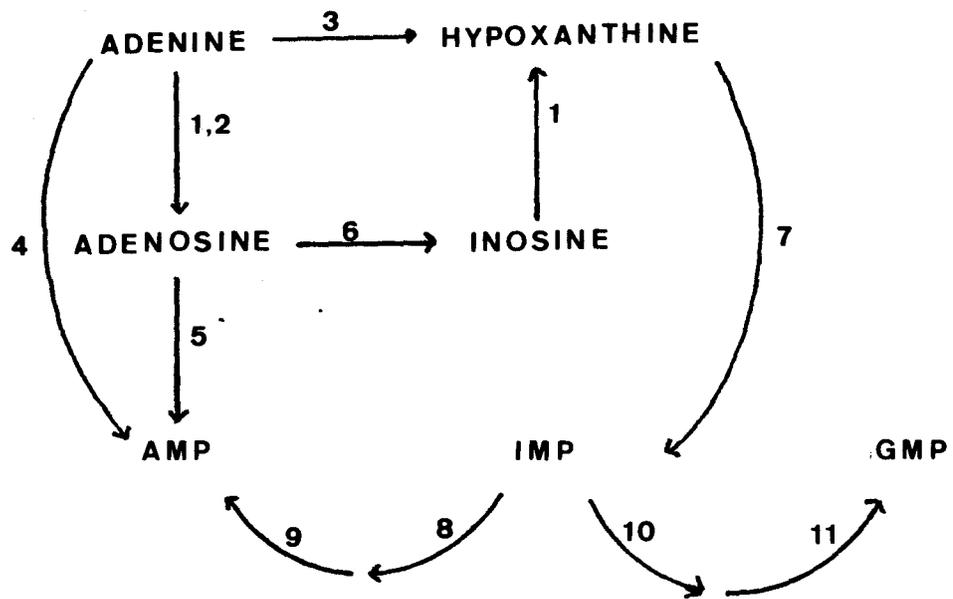
Five ml of YCB plus 0.3 mM adenine was innoculated with a loopful of 1540 and incubated for 24-36 hours at 30 °C. The cultures were centrifuged and the absorbance of the supernatant scanned over the range of 210-300 nm. Strains with adenine aminohydrolyase activity hydrolyze the adenine ( $\lambda_{\max}$  260 nm) to hypoxanthine ( $\lambda_{\max}$  248 nm).

Strain 1540 was found to possess the adenine aminohydrolyase mutation and was used in the in vivo isopentenyladenine metabolism studies.

Figure 24.

Proposed metabolism of adenine in S. cerevisiae.

1. purine nucleoside phosphorylase; 2. adenosine phosphorylase; 3. adenine aminohydrolyase; 3. adenine phosphoribosyltransferase; 5. adenosine kinase; 6. adenosine aminohydrolase; 7. hypoxanthine-guanine phosphoribosyltransferase; 8. adenylosuccinate synthetase; 9. adenylosuccinate lyase; 10. IMP dehydrogenase; 11. GMP synthetase.



APPENDIX B

## APPENDIX B

### Isolation of Isopentenyladenine Metabolism Mutants and Isopentenyladenine Auxotrophs

After the presence of a cytokinin metabolizing enzyme was established by the in vivo cytokinin metabolism study, attempts to clone and possibly sequence the gene coding for the enzyme were planned by generating mutants that could not utilize isopentenyladenine as a source of the adenine ring.

We tried to isolate from an adenine auxotroph mutants which lacked the cytokinin metabolizing enzyme. With DNA from wild-type yeast, we would construct a pool of recombinant plasmids using an appropriate yeast vector, transform the mutant, and screen for transformants that would again grow on isopentenyladenine. Any clones which grew would be isolated, the DNA extracted and the recombinant plasmid isolated. The yeast DNA would then be cleaved from the vector and characterized by restriction analysis. This proposed cloning procedure was never attempted since we were unable to obtain a mutant which was not able to metabolize isopentenyladenine (see below).

We attempted to induce the mutation with ethylmethane-sulfonate (EMS) by the method reported by Fink (1970). The EMS treated cells were screened for the absence of the isopentenyladenine metabolizing enzyme using nystatin to

enrich for the mutants (Snow, 1966). After cells of an *ade-4* strain were treated with EMS, they were grown in medium containing isopentenyladenine and nystatin. Nystatin kills growing yeast cells, but not those which remain static in the culture. Therefore, nystatin should selectively kill all cells which can metabolize isopentenyladenine and grow in the nystatin containing medium. Survivors were plated out on YPD medium. The colonies which appeared on the YPD medium were then tested for the inability to grow on isopentenyladenosine by dropping suspensions on isopentenyladenine-containing medium and checking for the absence of growth.

Media and SolutionsMinimal + Adenine

2% glucose  
 0.002% adenine  
 0.67 M YNB

Minimal + Isopentenyladenine

2% glucose  
 0.002% isopentenyladenine  
 0.67 M YNB  
 0.1% DMSO

Minimal without

$(\text{NH}_4)_2\text{SO}_4$   
 2% glucose  
 0.002% adenine or  
 isopentenyladenine  
 1 ml of 1%  $\text{CaCl}_2 \cdot \text{H}_2\text{O}$   
 1 ml vitamin and salt  
 solution\*  
 1 ml potassium phos-  
 phate buffer\*\*

Total volume = 100 ml

\* Vitamin and Salt  
Solution

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	50 g
$\text{NaCl}$	10 g
Pyridoxine	40 mg
Thiamine	40 mg
Pantothenate	40 mg
Inositol	200 mg
Biotin	0.2 mg
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	5 mg
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	7 mg
$\text{H}_3\text{BO}_3$	1 mg
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	1 mg
KI	1 mg

Total vol. = 1 liter of 100X

\*\* Phosphate Buffer

$\text{KH}_2\text{PO}_4$	87.5 g
$\text{K}_2\text{HPO}_4$	12.5 g
Total vol. = 1 liter	

Nystatin

1 mg of nystatin in  
 1 ml of 95% ethanol  
 diluted to 10 ml with  
 distilled water

ProcedureDay 1

The ade-4 cells were inoculated into 10 ml of YPD liquid medium and shaken in a 30 °C water bath overnight.

Day 2

The cells were harvested by centrifugation at 1500 rpm for 10 minutes and washed once. The cells were then resuspended in 10 ml of 0.1 M sodium phosphate buffer pH 8 and 0.3 ml EMS was added. the solution was vortexed and incubated at 30 °C for 50 minutes without shaking. The cells were washed three times and resuspended in 9 ml of liquid minimal medium supplemented with adenine and placed in a shaker bath for two days at 30 °C. EMS treatment resulted in a 88-92% kill of the exposed cells.

Day 4

One ml of the culture was removed and washed once. The cells were resuspended in 1 ml of minimal medium without ammonium sulfate + adenine and shaken overnight at 30 °C.

Day 5

The cells were centrifuged and resuspended in 0.9 ml of minimal medium with ammonium sulfate + isopentenyladenine. The culture was incubated at 30 °C for 6 hours. 0.1 ml of the 100 g/ml nystatin solution added and the culture incubated for one hour. After washing twice with water the cells were resuspended in 1ml of minimal medium + adenine. The first nystatin treatment killed 99% of the cells.

Day 7

The culture was washed twice and resuspended in 1 ml of minimal medium + isopentenyladenine without ammonium sulfate. The culture was centrifuged and resuspended in 0.9 ml of minimal media with ammonium sulfate + isopentenyladenine. After shaking for 6 hours at 30 °C, 0.1 ml of nystatin was added.

Day 8

The culture was incubated with the antibiotic for 1.5 hours. The cells were washed twice and resuspended in 1 ml of water. Appropriate dilutions were made and 0.1 ml of each dilution was spread on a YPD plate. The second nystatin treatment resulted in a 93-99% kill rate.

Day 10

Colonies which formed on the YPD plates were checked for the ability to metabolize isopentenyladenine by dropping suspensions of the colonies onto isopentenyladenine-containing plates.

The mutant search described above was also carried out on wild-type S. cerevisiae and a strain which has no isopentenylated tRNA in order to find a strain which requires isopentenyladenine for growth.

The protocol used in these experiments was the same as that described above with the following changes:

1.) In the previous mutant search, isopentenyladenine was included in the minimal medium used the day before and the day of the nystatin treatment so that cells which could metabolize isopentenyladenine would grow and be killed. In these experiments, supplements were not needed in this media since the yeast used was not deficient in any substance needed for growth.

2.) In the first mutant search adenine was included in the growth minimal medium between the EMS and first nystatin treatments as well as between the two nystatin treatments. In the studies looking for an isopentenyladenine-requiring strain, isopentenyladenine was added to the growth medium.

3.) Growth periods after the EMS and first nystatin treatment were lengthened from 2 days to 4 days to give the surviving cells an opportunity to grow before the nystatin treatment.

All attempts to isolate yeast mutants unable to metabolize isopentenyladenine to adenine or mutants which required isopentenyladenine for growth were unsuccessful. After testing a large portion of colonies that survived the EMS and nystatin treatments for the inability to grow on isopentenyladenine or the isopentenyladenine requirement, none appeared to be the desired mutant.

It is possible that the colonies that survived was that small percentage of cells that does not get killed by the

EMS and nystatin exposures - neither substance kills 100% of the cells. Another possibility is that the cells were mutated by the EMS and selected for by nystatin, but that the mutations reverted to the wild-type by the time the cells were tested. Another possibility is that the isopentenyladenine requiring strains and those unable to metabolize isopentenyladenine don't survive - perhaps these mutations are lethal to the cells.

APPROVAL SHEET

The thesis submitted by Carol Anne Van Kast has been read and approved by the following committee:

Dr. Howard M. Laten, Director  
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Assistant Professor, Biology, Loyola

Dr. Walter Tabachnick,  
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The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

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

April 14, 1986

Date

Howard M. Laten

Director's signature