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The De Novo Biosynthesis of Cytokinins by Cell-Free Extracts of Baker's Yeast

Debra A. Tonetti
Loyola University Chicago

THE *DE NOVO* BIOSYNTHESIS OF CYTOKININS BY CELL-FREE EXTRACTS OF BAKER'S YEAST

by

Debra A. Tonetti

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This thesis is dedicated to the memory of my cousin,

Donald J. Lencioni

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VITA

Debra A. Tonetti was born in 1958 in Chicago, Illinois to Mario and Josephine Tonetti.

In 1976, she completed her secondary education at Riverside-Brookfield High School, and then attended Triton Community College for one year. In 1977, Debra transferred to Northern Illinois University in DeKalb, where in 1980 she completed a Bachelor of Science degree in biology with a chemistry minor.

From 1980 to 1983, Debra was employed at Loyola University's Stritch School of Medicine in the Biochemistry Department where she was a Research Assistant in the laboratory of Mary Druse Manteuffel, Ph.D.

She began her graduate study in 1982 in the Department of Biology at Loyola University Lake Shore Campus. She conducted her masters research in the laboratory of Howard M. Laten, Ph.D. and was awarded a Teaching Assistantship.

In August of 1984, Debra was accepted into the doctoral program in Biochemistry at the Loyola University Medical Center and was awarded a Basic Science Fellowship.

Debra is a member of the American Association for the Advancement of Science.

Publications

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ABBREVIATIONS

Ade	Adenine
Ado	Adenosine
5'-AMP	Adenosine 5'-monophosphate
3',5'cAMP	3', 5'-Cyclic adenosine monophosphate
cpm	counts per minute
Da	Dalton
d^2-iPP	d^2-isopentenyl pyrophosphate
d^3-iPP	d^3-isopentenyl pyrophosphate
GLC	Gas liquid chromatography
Gua	Guanine
GMP	Guanosine monophosphate
HPLC	High Performance Liquid Chromatography
I^6Ade	Isopentenyladenine
I^6Ado	Isopentenyladenosine
I^6AMP	Isopentenyladenosine monophosphate
Ino	Inosine
Leu	Leucine
rpm	revolutions per minute
Ser	Serine

CHAPTER I

RATIONALE

The biosynthetic route for the production of free cytokinins remains an unresolved issue. Although de novo biosynthesis has been demonstrated in tobacco and slime mold (Chen & Melitz, 1979; Nishinari & Syono, 1980b; Taya et al., 1978), the exact contribution of this pathway and the tRNA-dependent pathway is as yet unknown.

The identification of de novo biosynthesis of cytokinins in non-plant systems is of considerable interest in view of the effects of free cytokinins on mammalian tissues. It is the intent of this study to provide direct evidence for the de novo pathway in the yeast Saccharomyces cerevisiae. This laboratory has previously reported the existence of free isopentenyladenosine (I6Ado) in a yeast mutant deficient in isopentenylated tRNA (Laten & Zahareas-Doktor, 1985). The present study focuses on the development of an assay for the detection of an enzyme activity responsible for the tRNA-independent biosynthesis of cytokinins. This goal was facilitated by the use of a mutant S. cerevisiae strain, mod 5-1, which lacks the tRNA-d2-isopentenyltransferase, thus eliminating a major competitor for substrates.

CHAPTER II

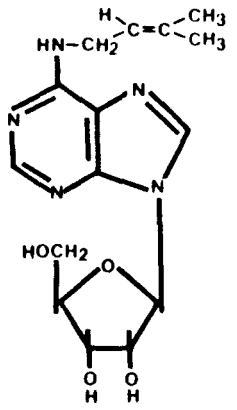
REVIEW OF RELATED LITERATURE

Cytokinins--Plant Growth Hormones

Cytokinins are a collection of molecules first identified in plants as growth factors which induce cell division. Structurally they are defined as N^6-substituted adenine derivatives. A correlation between structure and function of these molecules has been sought by the use of bioassays. Typically, the detection of cytokinin activity is measured utilizing a bioassay which is based on *in vitro* growth of plant tissue. For example, the tobacco bioassay (Linsmaier & Skoog, 1965) is capable of detecting concentrations in the nanomolar range. The most potent naturally occurring cytokinins appear to be N^6-isopentenyladenine (I^6Ade) derivatives. Figure 1 illustrates some common naturally occurring cytokinins. Apparently stringent N^6 side chain requirements must be met since optimal activity occurs with an isopentenyl group; activity decreases precipitously with changes in the n-alkyl chain length by about ten-fold for each C-atom added or subtracted (Skoog & Schmitz, 1979). Extensive studies have been done to delineate specific structure-activity relationships (Chen *et al.*, 1975; Hecht *et al.*, 1975; Henderson *et al.*, 1975). In

Figure 1. Structures of some naturally occurring cytokinins: a. Isopentenyladenosine; b. trans-Zeatin; c. Isopentenyladenine; d. Zeatin Riboside; e. cis-Zeatin

a.



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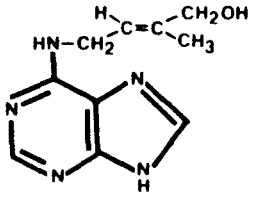

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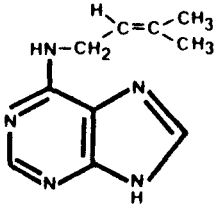

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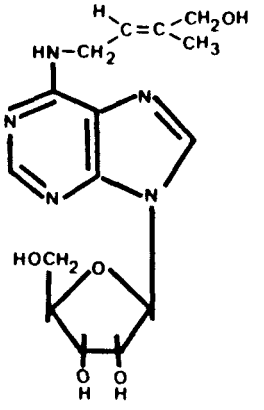
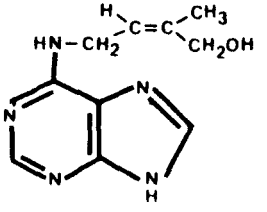


e.



spite of these studies, it appears that one specific structural property cannot be correlated with cytokinin activity. Skoog and Schmitz (1979) have proposed that it is the integrated properties of the molecule which act in concert to determine the ultimate biological activity.

The first compound discovered to cause cell division was termed kinetin (Miller et al., 1956) and has since been replaced by the general term cytokinin (Skoog et al., 1965). Since their initial discovery, a multitude of effects have been attributed to cytokinins. Currently it is known that these molecules, interacting with other plant hormones (auxins and gibberellins), influence differentiation of tissues, organogenesis, leaf growth, apical dominance, development of stolons, dormance and germination of seeds, and reproductive development (Skoog & Schmitz, 1979). Correlations have been drawn to the animal hormone system in that cytokinins are thought to be synthesized in specific areas and then translocated to target organs. Sites of cytokinin biosynthesis in plants are thought to include root tips, the developing seed, and the developing bud (Letham, 1978).

At the molecular level, cytokinins appear to stimulate protein synthesis at the transcriptional and translational level. Apparently cytokinins can either activate polyribosomes or stimulate ribosomes to form polyribosomes thereby enhancing the translational process (Szweykowska et al., 1981).

Direct evidence demonstrated increased RNA synthesis in a cell-free system. Addition of cytokinins to a mixture of DNA from pea buds, RNA polymerase and a receptor protein isolated from chromatin increased the rate of RNA synthesis (Matthysse & Abrams, 1970).

Analogous to the animal hormone system, cytokinin receptors have been identified in plants. Although several investigators have characterized binding parameters for these proteins, their physiologic importance has not as yet been explained. The first soluble cytokinin binding protein, CBF-1, was isolated from wheat germ ribosomes which exhibited both high affinity and specificity (Fox & Erion, 1975). This study indicated that only one-third to one-fourth of the binding protein was associated with the ribosomes. Other investigators report binding moieties apparently identical to CBF-1 (Moore, 1979; Poly & Davis, 1978). These studies report a lesser amount of binding protein in association with the ribosomal fraction. Interestingly, the cytokinin concentrations supporting growth in vivo are approximately ten-fold less than the reported Kd.

Isopentenyladenosine in tRNA

In addition to their occurrence as plant growth factors, cytokinins have been identified as modified bases in tRNA. tRNAs contain a large number of modified bases. The modifications are most common in the single-stranded

loops. A purine base adjacent to the 3' end of the anticodon is commonly hypermodified, and many of these bases have cytokinin activity. Hypermodifications at this position in tRNA are known to occur in all organisms yet examined including bacteria, plants, and animals (Hall, 1971). A further generalization restricts cytokinin moieties to those tRNA species which respond to codons beginning with uridine (Letham & Palni, 1983). Two studies which examined the distribution of cytokinins in different yeast and E. coli tRNA species (Armstrong et al., 1969a; 1969b) contributed evidence for this generalization. S. lactis and S. cerevisiae tRNA was fractionated on DEAE-cellulose and then hydrolysed to nucleosides for use in the tobacco bioassay. Utilizing this protocol, these investigators identified cysteine tRNA as a cytokinin-containing tRNA species. Those yeast tRNA species which correspond to codons beginning with G (alanine, aspartate, glutamate, glycine, and valine tRNA's) were found not to contain cytokinins. Previous studies identified a serine tRNA in rat liver (Staehelin et al., 1968), tyrosine, tryptophan, cysteine, phenylalanine, leucine, and serine tRNA's in yeast (Armstrong et al., 1969b) and tyrosine tRNA from yeast (Madison et al., 1967) to contain cytokinins that correspond to codons with U in the first position.

The parent nucleoside, isopentenyladenosine (I^6Ado), and its many derivatives appear to exhibit some species

specificity (Lethan & Palni, 1983). I^6Ado is the only cytokinin which occurs in yeast and animal tRNA, as well as in most organisms. Bacteria possess mainly methylthio derivatives. Hydroxylated derivatives such as cis- and trans-ribosylzeatin seem to predominate in plants. One exception to this is evident in the plant pathogenic bacteria, Agrobacterium tumefaciens. This pathogen induces tumor or gall formations in plants which secrete cytokinins. It has been found that this bacterium contains the hydroxylated derivative, ribosylzeatin in its tRNA (Cherayil & Lipsett, 1977) and primarily occurs in tRNA-ser and tRNA-leu (Morris et al., 1981). Morris et al. suggest that this plant-associated prokaryote has acquired genetic material from the host.

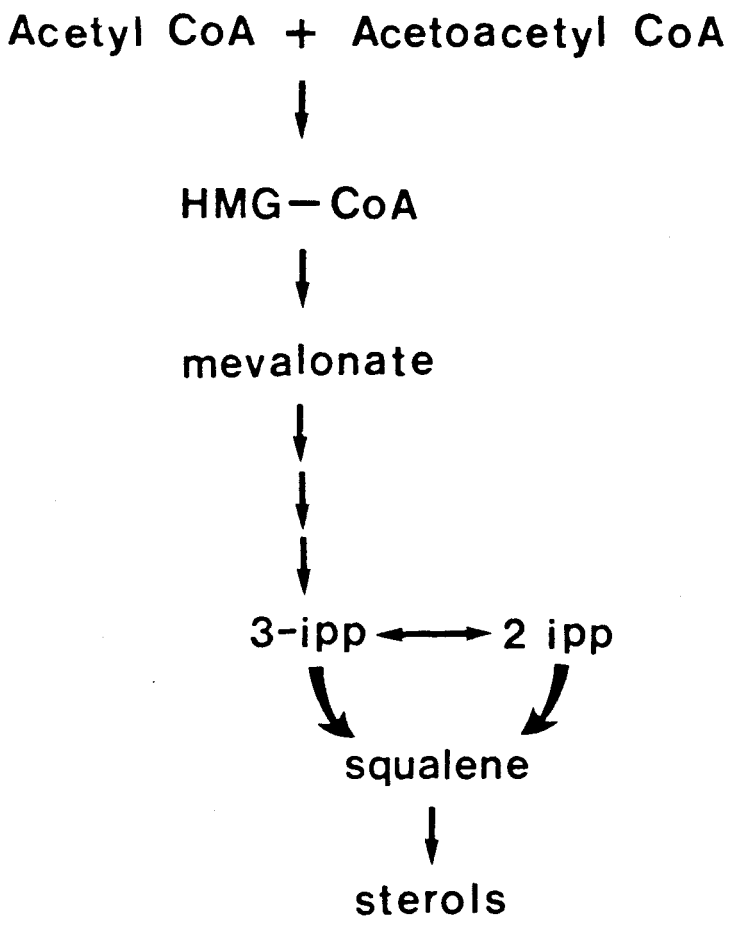
It is yet unclear whether the presence of cytokinins in tRNA is related to the growth-promoting function. A relationship has been established between tRNA-associated cytokinins and protein synthesis. More specifically, studies indicate that a modified base adjacent to the 3' end of the anticodon facilitates binding of the charged tRNA to the mRNA-ribosome complex. Fittler and Hall (1966) were able to selectively modify the I^6Ado component of yeast seryl-t-RNA with iodine treatment. Their results showed that this chemical modification did not alter amino acid acceptor function, but did interfere with the association between tRNA and the mRNA-ribosome complex in vitro. This finding was supported by Gefter and Russell (1969) using

E. coli. Using a transducing bacteriophage, phi 80, the synthesis of three forms of suppressor tyrosine tRNA occurred, differing only in the extent of modification of the base adjacent to the anticodon. The fully modified form was able to support in vitro protein synthesis and exhibited normal ribosome binding. The form lacking the modification was inefficient in in vitro protein synthesis and ribosome binding ability was diminished. It has been suggested that hypermodification of the base adjacent to the anticodon prevents misreading of the codon and insures the single-stranded conformation of the anticodon loop (Parthasarathy et al., 1974). Also in E. coli it appears that the thiomethylated I^6Ado containing tRNA binds better to ribosomes than I^6Ado (Hoburg et al., 1979). It has been shown that the loss of hypermodification at this position does not effect protein synthesis at the level of aminoacylation (Laten et al., 1980).

The isoprene side chain of I^6Ado containing tRNA is derived from the sterol biosynthetic pathway (Fig. 2). Several investigators were involved in elucidating the isopentenylation of tRNA (Fittler et al., 1968a, 1968b; Holtz & Klambt, 1975; Kline et al., 1969; Murai et al., 1975; Peterkofsky, 1968; Rosenbaum & Gefter, 1972). Fittler et al. (1968a) established the precursor relationship of mevalonate to the d^2-isopentenyl group in tRNA of Lactobacillus. Using mevalonate-requiring strains grown in the

Figure 2. The sterol biosynthetic pathway. d^2-ipp is an intermediate in this pathway and serves as the donor substrate in the isopentenylation of tRNA.

STEROL BIOSYNTHETIC PATHWAY



presence of ^{14}C-mevalonate, all the tRNA-associated radioactivity could be accounted for by the isopentenyl group of I^{6}Ado. Crude enzyme extracts from yeast and rat liver were isolated which catalyzed the incorporation of mevalonate or d^2-isopentenylpyrophosphate (d^2-iPP) into preformed tRNA *in vitro* (Fittler *et al.*, 1968b). This study demonstrated that isopentenylation occurs at the macromolecular level and not at the mononucleotide level. Other studies indicated that a specific adenosine residue of tRNA serves as the acceptor of the d^2-isopentenyl group in yeast. Treatment of tRNA with permanganate cleaves the d^2-isopentenyl group from tRNA, yielding a suitable substrate for tRNA-d^2-isopentenyltransferase. Iodine treatment prevents permanganate from reacting with the isopentenyl group, and the tRNA will not accept an isopentenyl group in the transferase reaction. The donor molecule in the reaction was found to be exclusively d^2-iPP and not d^3-ipp (Kline *et al.*, 1969). This isomerization is catalyzed by isopentenylpyrophosphate isomerase (EC 5.3.3.2) which was initially isolated from yeast (Agranoff *et al.*, 1968). Other investigators have described this enzyme in porcine liver (Holloway & Popjak, 1967, 1968; Shah *et al.*, 1965) and avian liver (Sagami & Ogura, 1983).

The discovery and isolation of naturally occurring I^{6}Ado-deficient tRNA facilitated the study of the tRNA-d^2-isopentenyltransferase reaction as well as the pathway leading to free I^{6}Ado levels (Gefter & Russell, 1969; Laten

et al., 1980). In addition to tRNA as substrate, Holtz and Klambt (1978) have demonstrated isopentenylation of oligonucleotides by tRNA-d^2-isopentenyltransferase from Zea mays L., albeit at a reduced preference for the enzyme.

Biosynthesis of Free Cytokinins

Apart from their occurrence as components of tRNA, free cytokinins are found in plants (Letham & Palni, 1983), some plant pathogenic bacteria (Palni et al., 1983) slime mold (Ihara et al., 1984), yeast (Laten & Zahareas-Doktor, 1985), and plant-associated insects (Elzen, 1983). In recent years much attention has been focused on the elucidation of the biosynthetic pathway responsible for the production of free cytokinins. Two pathways have been postulated. Since cytokinins are components of tRNA, one theory postulates that the natural turnover of tRNA provides the free cytokinin pool. The alternate hypothesis is that a de novo pathway independent from tRNA turnover, is responsible for the synthesis of the free forms. A plethora of direct as well as indirect evidence has accumulated in favor of both pathways. It appears likely that both mechanisms may be integral in contributing to the free cytokinin pool, however each organism may differ in the mechanism acting as the major pathway. For example, experiments conducted by Helbach and Klambt (1981) suggested that tRNA and oligonucleotide turnover is the only active pathway in the bacteria

Lactobacillus acidophilus. By supplying this mevalonate auxotroph with ^{14}C-mevalonate in a pulse-chase experiment, these researchers were able to specifically label the cytokinin-containing nucleic acids. The appearance of labelled free cytokinins was monitered as well as the incorporation into tRNA. Following the time course of pulse labelled tRNA, it was apparent that as the tRNA turned over, the label was found to increase in the free cytokinins. These results are in agreement with previous studies which determined the half-life of L. acidophilus tRNA to be three hours (Helbach et al., 1978).

Much interest has been focused on determining the pathway of free cytokinin production in higher plants since there is an associated hormone action. An early study which attempted to clarify whether a de novo pathway existed, utilized a cytokinin-autonomous line of tobacco tissue (Dyson & Hall, 1972). Cytokinin-dependent tobacco tissue and the autonomous line were similar in that both contained I^{6}Ado and ribosylzeatin within the tRNA, but differed in that the autonomous line contained an appreciable level of I^{6}Ado in the free state. If tRNA turnover provides the cell with free cytokinins, why is the cytokinin-dependent tissue devoid of any free I^{6}Ado?

Again employing the autonomous tobacco tissue, Chen et al. (1976) were able to provide evidence for the tRNA-independent biosynthesis of cytokinins. ^{14}C-Adenine and a

$^{14}C/^{3}H$-labelled adenosine analog were supplied. It was found that the tissue was capable of synthesizing labelled $I^{6}Ado$ and its derivatives. It was unlikely that the $^{14}C/^{3}H$-$I^{6}Ado$ resulted from tRNA turnover since the $^{14}C/^{3}H$ adenosine analog was shown not to be incorporated into tRNA. Characterization of labelled free cytokinins in a cytokinin-autonomous tobacco cell line when ^{3}H-adenine was supplied was accomplished by Sephadex LH20 chromatography and reverse phase HPLC (Burrows, 1978). The major radioactive cytokinins produced included $I^{6}Ade$, $I^{6}Ado$, trans-ribosylzeatin, and trans-zeatin. Since the cis isomer of zeatin and the oxidized form t-$io^{6}Ado$ are found in tRNA, it was improbable that the free cytokinins were tRNA degradation products. A similar follow-up qualitative study compared cytokinins of tRNA and free forms from Lupinus luteus L. seeds and Populus x robusta schneid leaves. Once again the free forms differed from the tRNA-associated species. Although these results do not preclude a tRNA turnover pathway, they strongly support the importance of a de novo pathway.

The first piece of direct evidence for the de novo pathway came from the slime mold, Dictyostelium discoideum (Taya et al., 1978). These cells contained a large quantity of $I^{6}Ade$ which is thought to be a precursor to the spore germination inhibitor, discadenine. This study, utilizing a cell-free system, demonstrated that 5'-AMP was the direct precursor of free cytokinins. The reaction mixture consisted

of ^{14}C-iPP, crude enzyme extract, boiled non-dialysed supernatant (cell extract) in buffer at pH 7.5. After a one hour incubation, the mixture was extracted with ethyl acetate, chromatographed on silica gel, and scanned with a radiochromatogram scanner. A radioactive product was identified as I^6Ade. This experimental design implicated that a heat-stabile dialyzable substance was the acceptor of the isopentenyl group. Various compounds such as 5'-AMP, ADP, ATP, and 3',5'-cAMP were tested, and 5'-AMP was found to be the direct acceptor.

Additional direct evidence in cytokinin-autotrophic tobacco tissue cultures was reported by Chen and Melitz (1979). In this study, the investigators isolated an enzyme system capable of catalyzing the synthesis of I^6Ado-5'-P from d^2-iPP and 5'-AMP. I^6Ado and I^6Ade were also detected among the reaction products, but only when a crude enzyme preparation was used. The authors suggested these molecules were degradation products modified by nucleosidases and phosphatases. Chen (1982) reported dual-labelled products when (2,8-^3H)Ade and (1-^{14}C) d^3-iPP were supplied in the culture medium. Analysis of these products by GLC revealed I^6Ado, I^6Ade, zeatin, and ribosylzeatin. Formation of the hydroxylated derivatives was attributed to microsomal mixed function oxidases.

d^2-iPP:5'-AMP-d^2-isopentenyltransferase was further purified approximately 48-fold when compared to the crude

extract, with a specific activity of 78 nmol I^6Ado-5'-P/min/mg protein. The molecular weight of partially purified enzyme was estimated by Sephadex G-200 gel filtration to be 52,000 +/-2000. In characterizing the enzyme, optimal activity was achieved at pH 7.0, with an absolute requirement for Mg^{2+} ion. The enzyme is specific for d^2-iPP and 5'-AMP. Ado, Ade, cAMP, GMP, Gua, and Ino could not be substituted as the acceptor molecule. Chen concluded that although ^{14}C-d^2-iPP and ^3H-adenine condensed to yield $^{14}C/^3H$ dual-labelled cytokinin products <u>in vivo</u>, adenine was not a substrate for the isopentenyltransferase <u>in vitro</u>. He speculated that other enzymes may be involved in the <u>de novo</u> biosynthesis of cytokinins. The alternate explanation was that the dual-labelled <u>in vivo</u> products may be the result of incorporation into tRNA that subsequently turned over.

Ihara <u>et al</u>. (1984) later purified d2-isopentenyl-pyrophosphate:5'-AMP d^2-isopentenyltransferase 6800-fold from <u>D. discoideum</u>. 5'-AMP was shown to be the preferred substrate, however it was observed that ADP was 60-80% as effective as 5'-AMP. In comparing this enzyme to that isolated from tobacco (Chen, 1982) both similarities and differences were observed. Both enzymes have a requirement for Mg^{2+} ion, and a pH optimum of 7.0. However the slime mold enzyme demonstrated higher enzyme activity with Zn^{2+} and Mn^{2+}. The slime mold enzyme molecular weight was

estimated by Sephadex G-100 gel filtration to be 40,000 +/- 2000 Da, which is close to the 52,000 +/- 2000 Da estimate made for the tobacco enzyme. The Km of the tobacco cell enzyme for 5'-AMP is 47 times higher than the reported Km for slime mold.

As eluded to previously, plant pathogenic bacteria induce the formation of tumors or crown-gall disease, although the bacteria are not present in these tissues. These galls produce large amounts of cytokinins. Stuchbury et al. (1979) observed that radiolabelled adenine was rapidly converted to ribosylzeatin phosphates, zeatin and ribosylzeatin in vivo. Considering the tRNA in higher plants was estimated to have a half-life of 3-5 days, (Klemen & Klambt, 1974) a pathway involving tRNA incorporation prior to release seemed unlikely.

In an attempt to discern the maximum contribution of tRNA turnover to free cytokinin levels, Barnes et al. (1980) grew potato cells in liquid culture in the presence of mevalonic acid lactone-(2-^{14}C) and calculated the relative rate of cytokinin release by tRNA turnover. It was concluded that tRNA turnover could account for no more than 48% of the free cytokinin pool. ^{14}C-adenine pulse-chase experiments using roots from the bean, Phaseolus vulgaris, indicated that primary incorporation occurred in tRNA and secondarily in oligonucleotides and mRNA (Maass & Klambt, 1981).

Nishinari and Syono (1980b) reported findings

similar to Taya et al. (1978) and Chen and Melitz (1979) demonstrating cell-free biosynthesis of cytokinins in cultured tobacco cells. However, in addition to 5'-AMP, they found that adenine and adenosine also served as substrates for the enzyme. Subsequently, a pathway was proposed involving ^{14}C-adenosine incorporation into butanol soluble cytokinins in vivo in synchronously dividing cells (Nishinari & Syono, 1980c) and was compared to the previous in vitro study. The results indicated discrepancies between the in vitro and in vivo pathways. These authors currently are working to clarify these differences. It seems likely that in vivo, rapid interconversion of metabolites occurs due to the presence of other enzymes. This would explain the inability to detect rapidly converting intermediates.

Palni et al. (1983) were able to determine the significance of nucleotides in V. rosea crown-gall tissue in the biosynthesis of free cytokinins. Using anion exchange chromatography, distinct separation of the mono-, di-, and triphosphates was achieved. These studies led to the conclusions that trans-zeatin monophosphate was the main active nucleotide, that biosynthesis probably occurred at the mononucleotide level, and that it was tRNA-independent.

Recently, the gene responsible for cytokinin biosynthesis was cloned from Agrobacterium tumefaciens, the crown-gall inducing bacterium (Barry et al., 1984). The virulence plasmid Ti of A. tumefaciens contains a small

region called the tmr locus which is associated with phytohormone metabolism. This region was cloned into E. coli cells using a plasmid. Expression was maximized using a strong E. coli promoter and confirmed by an isopentenyltransferase assay. The reaction was found to be specific for 5'-AMP and iPP. Adenosine could not be used as substrate. E. coli was suitable for this study since it has been shown to lack the d^2-isopentenyltransferase enzyme.

From the above discussion it is apparent that there is some ambiguity in the acceptor substrate for the isopentenyl group. Chen and coworkers (1979, 1982) report 5'-AMP to be the direct acceptor in tobacco tissue culture. Taya et al. (1978) and Ihara et al. (1984) both studying D. discoideum also report 5'-AMP as well as ADP to be involved. Nishinari and Syono (1980b), observed that 5'-AMP, adenine, and adenosine all are equally suitable substrates although this group reports differences between in vitro and in vivo studies. Palni et al.(1983) and Barry et al. (1984) also agree that the acceptor molecule is a mononucleotide. Van Staden and Forsyth (1984), attempted to clarify the role of adenine and adenosine in the pathway leading to free cytokinins in excised maize roots. It has been suggested that roots are sites of cytokinin biosynthesis (van Staden & Smith, 1978). This study showed that no incorporation of label could be found in the biologically active cytokinins when ^{14}C-adenine and ^{14}-adenosine was supplied to the media.

They suggested several possibilities

1. Adenine and adenosine may not serve as precursors.
2. Roots are unable to isopentenylate adenine or adenosine.
3. The isopentenyl side chain was not available in the experimental system.

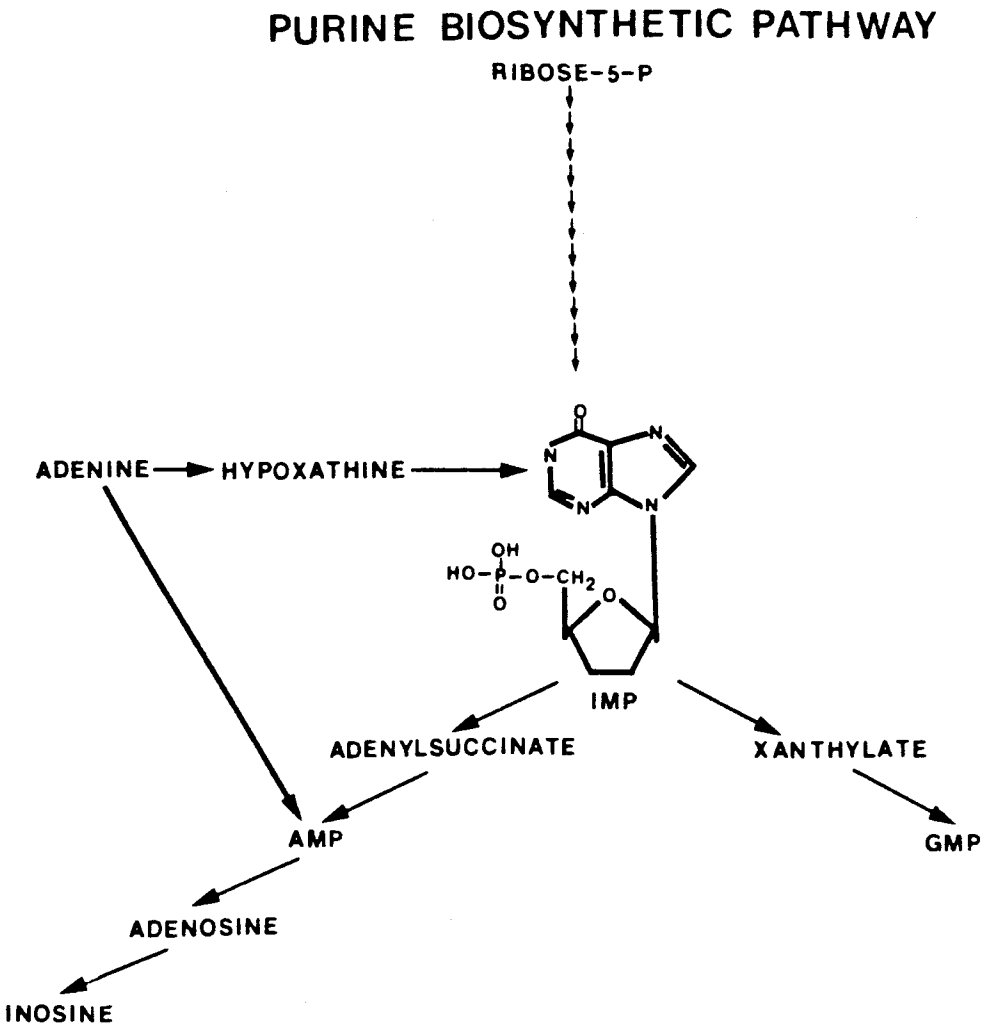
So it sppears that the identity of the direct acceptor molecule is still unclear. In reviewing the purine biosynthetic pathway (Fig. 3), it appears that only few molecules are likely candidates as the acceptor of the isopentenyl group.

Cytokinin Effects in Mammalian Cells

To date, the only established function of free endogenous cytokinins is that of potent growth stimulation in higher plants. The only non-plant systems so far shown to contain free cytokinin molecules are yeast (Laten & Zahareas-Doktor, 1985) and slime mold (Taya et al., 1978). The only identified function of these molecules is in slime mold, where I^6Ade has been identified as a precursor to the spore germination inhibitor, discadenine. The evidence that has accumulated strongly suggests that these molecules are not the products of tRNA turnover. Since these modified nucleosides are known components of mammalian tRNA, it is of interest whether any function can be associated with them after release following normal turnover.

It has been reported that I^6Ado stimulates mitosis

Figure 3. The purine biosynthetic pathway. Adenine, adenosine and AMP are the only structures which contain an amino nitrogen at position 9 on the purine heterocyclic ring. Precursors to these molecules are incapable of isopentenylation due to the presence of a keto group.


PURINE BIOSYNTHETIC PATHWAY
RIBOSE-5-P
ADENINE
HYPOXATHINE
IMP
ADENYLSUCCINATE
XANTHYLATE
AMP
GMP
ADENOSINE
INOSINE

in human lymphocytes at low concentrations (10-7 M) and inhibits at higher concentrations (10-6 M) (Gallo et al., 1969). These cells were pretreated with phytohemagglutinin which causes a morphological transformation yielding a cell capable of DNA synthesis, followed by mitosis in vitro. Without this treatment, in vitro studies of DNA synthesis and mitosis would not be possible. These authors propose that DNA synthesis inhibition may be due to I^6Ado competing with adenosine or ATP.

I^6Ado also exhibits anti-tumor activity. Several tumor types have been shown to be susceptible to I^6Ado treatment including trophoblastic tumor cells (Trewyn & Kerr, 1979) and lymphocytic leukemic cells (Grace et al., 1967). It has been postulated that I^6Ado competes with coenzymes containing adenosine for the enzyme binding site, and thus can inhibit a large number of metabolic enzymes (Tritsch, 1973).

I^6Ado has also been shown to elicit immunosuppressive effects (Hacker & Feldbush, 1969). More specifically, I^6Ado inhibited the incorporation of uridine into RNA and thymidine into DNA in phytohemagglutinin transformed rat spleen cells. Interestingly, when I^6Ado is conjugated to albumin and injected into rabbits, an unusually strong antibody response is elicited (Humanyun & Jacob, 1974). This response is in contrast to the relatively weak titres observed after immunization with the common nucleotide-albumin

conjugates. This result suggests that I^6Ado is not a normally circulating compound and is thus recognized as foreign. This finding is curious in view of the reported immunosuppressive effects of the free nucleoside.

A more recent study (Quesney-Huneeus et al., 1980) identifies I^6Ado and zeatin as mediators of DNA replication. It has previously been determined that mevalonate production is linked to maintaining normal cell growth, and that uncontrolled mevalonate synthesis is characteristic of all malignant and premalignant tumors in vivo. Quesney-Huneeus et al. (1980) demonstrated that I^6Ade and zeatin were 100 times more active in restoring DNA synthesis to cells which were blocked by compactin (HMG CoA reductase inhibitor), than mevalonate itself. More specifically, it was found that I^6Ade and zeatin were specific for S-phase DNA synthesis. Equally interesting is the finding that both I^6Ade and mevalonate can reverse inhibition of DNA synthesis induced by nalidixic acid. This finding suggests I^6Ade may be exerting regulatory effects on DNA gyrase, which is the enzyme that catalyzes the negative supercoiling of circular DNA in bacteria. In prokaryotes, DNA gyrase has been shown to be specifically inhibited by nalidixic acid. This study has important implications as to how I^6Ade may be involved in maintaining replicative homeostasis in the normal cell and in the pathophysiology of malignancy. These latter

results further confuse the mechanism of anti-tumor effects exerted by exogenously supplied I^6Ade.

CHAPTER III

MATERIALS AND METHODS

Growth of Cells

Two strains of Saccharomyces cerevisiae were employed, wild type (HL370) and mod 5-1 (HL430). The wild type cells were grown in liquid YPD medium (1.0% yeast extract, 2.0% dextrose, 2.0% peptone), SD + YE medium (0.67% yeast nitrogen base w/o amino acids, 2.0% dextrose, 0.1% yeast extract) or YPG medium (1.0% yeast extract, 2.0% peptone, 3.0% v/v glycerol). The mod 5-1 strain was grown in YPAD medium (YPD + 0.003% adenine). ^{14}C-adenine labeling experiments made use of wild type cells grown in SD + YE media, while YPG media was used for experiments testing mitochondrial expression. All other experiments utilized mod 5-1 cells grown in YPAD media. The cells were grown at 30 C in a shaking water bath to an optical density corresponding to mid-log phase on the growth curve. The cells were harvested by centrifugation at 5000 rpm in an SS-34 rotor for 20 minutes.

Cell Breakage

Buffer A (10 mM Tris-HCl, pH 7.5; 10 mM magnesium acetate; 6.0 mM KCl; 1.0 mM EDTA; 6.0 mM 2-mercaptoethanol;

25% glycerol, w/v; 25% polyvinyl polypyrrolidone, w/v) or buffer B (10 mM Tris-HCl, pH 8.0; 10 mM magnesium acetate; 60 mM KCl; 1.0 mM EDTA; 6.0 mM 2-mercaptoethanol; 10% glycerol, w/v) was added to the yeast paste in the ratio of 2.0 grams of buffer per gram wet weight of cells. The cells were then broken by using glass beads in a blender. The homogenization was carried out at 0-4 degrees C for 15 second intervals for a total time of 3.0 minutes.

The cellular debris was removed by centrifugation at 30,000 x g. The supernatant was retained and protein was assessed by the method of Lowry et al. (1951).

Isopentenyl Pyrophosphate Isomerase Preparation

A crude iPP isomerase enzyme preparation was obtained from wild type bakers yeast (Agranoff et al. 1960). The cells were broken using 2.0 M Tris buffer, pH 7.0. The cellular debris was removed as previously described. The resultant supernatant was exhaustively dialyzed against buffer C (0.02 M Tris-HCl, pH 7.5; 0.3 M KCl; 0.01 M $MgCl_2$; 0.1 M EDTA), and protein was determined by the method of Lowry et al. (1951).

A more purified enzyme preparation was prepared by Mrs. Ruth Timmons from pig liver & Popjak, 1967).

Isopentenyl Pyrophosphate Isomerase Enzyme Assay

Assay for the yeast enzyme was performed by the

method of Agranoff et al. (1960). The reaction mixture contained 3.0 umoles Mg Cl_2; 2.0 umoles Tris, pH 8.0; 0.1 umole ^{14}C-d3-iPP (57 mCi/mmol) and 1.0 mg. of protein, in a final volume of 0.5 ml. Aliquots were removed and the reactions were terminated by the addition of 6.0% TCA at 0, 10, 20, 40, 60, and 120 minutes.

The tubes were centrifuged in a clinical centrifuge and the supernatant was extracted with ether. An aliquot of the aqueous layer was placed on glass fiber discs, the discs were put into scintillation vials along with 5.0 ml of toluene based scintillation cocktail and counted in a Packard Tri-carb liquid scintillation counter. As d^3-iPP is converted to d^2-iPP the radioactivity decreases in the aqueous layer; therefore isomerase activity is detected by a decrease in counts as the reaction proceeds. Activity is expressed in nmol/min/mg protein.

AMP-d^2-Isopentenyltransferase Preparation

Approximately 3.0 grams of mod 5-1 cells were harvested and mechanically lysed in either buffer A or buffer B. After removal of the cellular debris by centrifugation, the supernatant was precipitated with ammonium sulfate. The fractions precipitating between 20-90% were collected by centrifugation at 20,000 x g for 20 minutes, resuspended in a small volume of buffer and dialyzed against three changes of the same buffer. A protein assay was

performed on the dialysate (Lowry et al., 1951), and the protein solution was concentrated to either 100 mg/ml or 10 mg/ml using Millipore CX-10 immersible ultrafilters. The enzyme preparation was kept at 0-4 degrees C throughout the procedure, and was used immediately in the enzyme assay (Fig. 4).

AMP-d^2-Isopentenyltransferase Assay

^{14}C-labelled d^3-iPP (57 mCi/mmole, Amersham) was incubated in either buffer D (7.0 mM Tris-HCl, pH 7.0; 20 mM $MgCl_2$; 35 mM $MnCl_2$; 3.5 mM 2-mercaptoethanol; 7.0 mM KF) or buffer E (20 mM Tris-HCl, pH 7.5; 5.0 mM magnesium acetate). At time zero, an iPP isomerase preparation from porcene liver or Baker's yeast was added to the reaction. At 15 minutes, the isopentenyl acceptor substrate was added (5'-AMP, adenine, or adenosine) as well as the isopentenyltransferase preparation (0.25-1.3 mgs). Isotopically labelled acceptor molecules included ^3H-5'-AMP (11.7 Ci/mmole, Amersham) or ^3H-Adenosine (41 Ci/mmole, Amersham). The reaction was terminated at various times (5, 10, 15, 30, 60, and 90 minutes) by the addition of one volume of ethanol or by cooling on ice. Controls were assayed in parallel using boiled isopentenyltransferase and/or iPP isomerase preparations. For specific experimental assay parameters, see Table 1.

Figure 4. Preparation of crude yeast isopentenyltransferase. The summarized procedure describes the method of crude enzyme preparation.

PREPARATION OF CRUDE YEAST
d^2-ISOPENTENYLTRANSFERASE

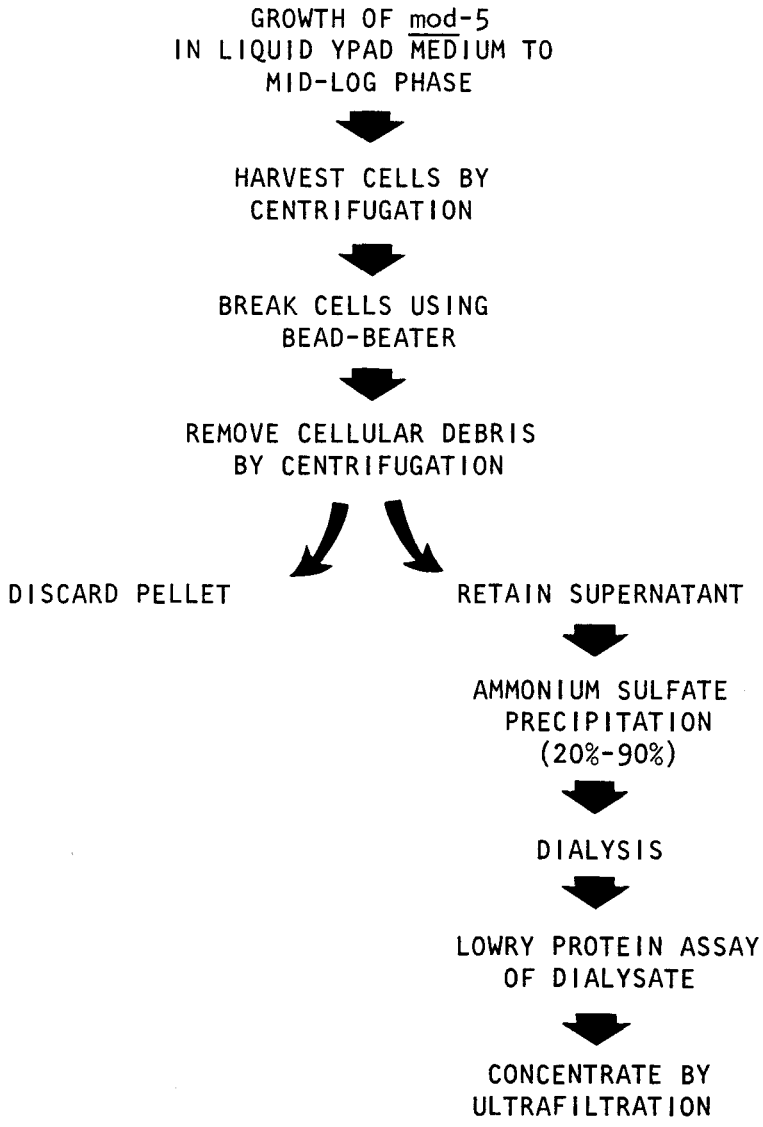


TABLE 1.--Isopentenyltransferase Assay Parameters. Each experiment (1-8) is listed with specific conditions describe
Abbreviations: CCFE--Crude cell--Free Extract; ASF--Ammonium Sulfate Fractionation; Wt BY--Wild Type Bakers Yeast; PL--P
Liver; BTP--Boiled Transferase Preparation; BIP--Boiled Isomerase Preparation

Experiment Number	Isopentenyl-transferase Enzyme Source	Level of Purification	mg Protein Isopentenyltransferase Preparation	Temperature Degree Centigrade	Buffer pH	Incubation Time	Ipp Isomerase Enzyme Source mg Protein	uCi 14c-13-Ipp (nmoles)	Cold Substrates (nmoles)	Labelled Substrates (nmoles)	Reaction Final Volume	Control
1	mod-5	CCFE	.025 mg	37 C	Chen pH 7.0	30 min	WT BY 40 ug	0.8 uCi 14 nmol	5'-AMP 100 nmol	--	0.1 ml	--
2	mod-5	ASF 20, 40, 60, 90%	.025 mg	37 C	Chen pH 7.0	30 min	WT BY 40 ug	0.8 uCi 14 nmol	5'-AMP 100 nmol	--	0.1 ml	BTP & BIP
3	mod-5	ASF 15-90%	.025 mg	37 C	Chen pH 7.0	30 min	WT BY 40 ug	0.8 uCi 14 nmol	5'-AMP 100 nmol	(^{3}H) 5'AMP .89 nmol	0.1 ml	BTP & BIP
4	mod-5	ASF 15-90%	1.3 mg	37 C 25 C	Chen pH 7.0 Taya pH 7.5	60 min	WT BY 40 ug	0.8 uCi 14 nmol	5'-AMP 100 nmol	(^{3}H) 5'-AMP .89 nmol	1.0 ml	BTP & BIP
5	mod-5	ASF 20-90%	1.0 mg	25 C	Taya pH 7.5	15, 30, 60 min	WT BY 40 ug	0.5 uCi 9.9 nmol	Ade, Ado AMP ADP, ATP, BNDS	(^{3}H) 5'-AMP .89 nmol	1.0 ml	BTP & BIP
6	mod-5	ASF 20-90%	1.0 mg	25 C	Taya pH 7.5	5, 15, 30, 60 min	PL 700 ug	0.4 uCi 7.9 nmol	Ado 50 nmol	^{3}H-Ado .024 nmol	1.0 ml	--
7	mod-5	ASF 20-90%	0.8 mg	25 C	Taya pH 7.5	30 min	PL 700 ug	1.14 uCi 20 nmol	Ade,Ado, 5'-AMP 200 nmol	^{3}H-Ado .024 ^{3}H-AMP .088	0.1 ml	BTP
8	WT	ASF 20-90%	.475 mg	25 C	Taya pH 7.5	10,30 min	PL 700 ug	1.14 uCi 20 nmol	Ado 5'-AMP 200 nmol	^{3}H-AMP .09 nmol ^{3}H-Ado .024 nmol	0.3 ml	--

Identification of Biosynthetic Products

Several schemes were employed in the identification of the biosynthetic products. These included:

(A) Anion exchange chromatography, followed by Sephadex LH-20 chromatography, and reverse phase high performance liquid chromatography (HPLC).

(B) Sephadex G-25 chromatography followed by reverse phase HPLC.

(C) Direct analysis on reverse phase HPLC.

Dowex anion exchange resin was swollen in aqueous .0025 N NaOH. The reaction mixture was applied to the top of the column (16 mm x 20 cm) at a flow rate of 15 ml/hr. The column was then eluted with a stepwise KCl gradient (0.0 M KCl, 0.1 M KCl, 0.3 M KCl; in .0025 N NaOH) and fractions were collected (3.0 ml/fraction). 1.0 ml aliquots of each fraction were placed in scintillation vials with 10 ml of Aquasol (VWR) and counted in a Packard Tri-Carb liquid scintillation counter. Radioactive fractions were combined and further chromatographed on Sephadex LH-20.

A small aliquot of the combined radioactive fractions (>500 CPM) was applied to a Sephadex LH-20 column (1.5 cm x 28.0 cm). The radioactive sample was simultaneously chromatographed with authentic cytokinin standards in 33% aqueous ethanol at a flow rate of 12.0 ml/hr. The column was monitered using a UV spectrophotometer at 254 nm and 1.0 ml fractions were collected. Aliquots from each fraction were

removed for liquid scintillation counting.

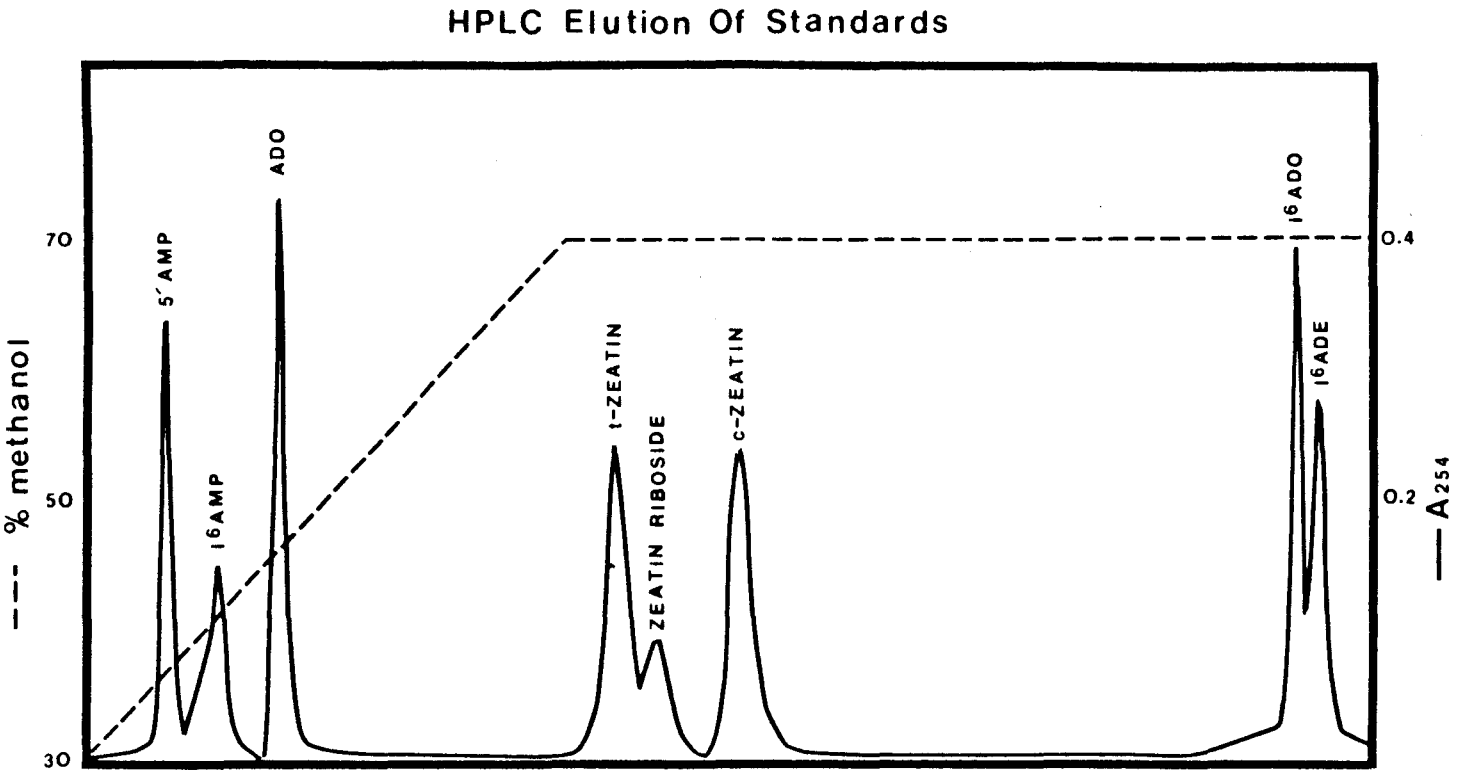
In some instances, a Sephadex G-25 column was used in place of anion exchange chromatography. The eluent used was distilled deionized water and the column dimensions were 10 mm x 10 cm. The reaction mixture was loaded directly onto the column and eluted at a flow rate of 14.4 ml/hour. Fractions were collected and counted.

Reverse phase HPLC was utilized for final identification. Prior to HPLC injection, samples from Sephadex LH-20 and Sephadex G-25 were concentrated either by rotoevaporation or under a stream of nitrogen gas. Injection volumes varied between 25 ul and 100 ul. The mobile phase was aqueous methanol using either isocratic elution (55%) or a linear gradient, (30-70%) aqueous methanol. The eluent was monitored at 254 nm and the flow rate ranged between 0.5 and 1.0 ml/minute. Fractions were collected directly into scintillation vials. See Figure 5 for a typical elution profile.

^{14}C-Adenine Labelling

Wild-type yeast cells were grown in SD + YE media in the presence of 10 uCi ^{14}C-adenine (53 Ci/mmole). A 10 ml liquid culture was inoculated at 0.16 O.D. The cells were harvested after 2.0 hours and 6.0 hours. 3.0 ml aliquots were removed at each time point, centrifuged in a SE-12 rotor at 8000 RPM for 20 minutes, and washed twice

Figure 5. RP-HPLC Elution Profile. The column was eluted with a 30-70% aqueous methanol gradient at a flow rate of 1.0 ml/min. The following standards were chromatographed: 5'-AMP; I^6AMP; Adenosine; t-Zeatin; Zeatin Riboside; c-Zeatin; I^6Ado; I^6Ade. The eluent was monitored by UV at 254 nm.


HPLC Elution Of Standards
--- % methanol
70
50
30
5'AMP
I6AMP
ADO
t-ZEATIN
ZEATIN RIBOSIDE
c-ZEATIN
I6ADO
I6ADE
0.4
0.2
— A254

with cold water. A final concentration of 1.0 ug zymolyase/mg yeast cells was obtained using a solution of 0.1 mg zymolyase/ml Scott's buffer (50 mM KH_2PO_4, 10 mM 2-mercaptoethanol; pH 7.5). The cell suspension was transferred to microcentrifuge tubes and incubated for 3.0 hours at 30 C in a shaking water bath. NP-40 was added to a final concentration of 10% and the suspension was incubated an additional 15 minutes. The cells were examined under the microscope to confirm cell lysis prior to microcentrifugation for 15 minutes. The supernatant was retained and loaded onto a Waters C18 Sep-Pak cartridge. The column was washed once with 4.0 ml of water, and then with 4.0 ml of methanol. The methanol wash was collected in a 50 ml round bottom flask, rotoevaporated to dryness, resuspended in a small volume of methanol, and injected into the HPLC. The amount of radioactivity was checked before and after rotoevaporation.

CHAPTER IV

RESULTS

Isopentenyl Pyrophosphate Isomerase Activity

The activity of the yeast iPP isomerase was calculated to be 0.00139 nmol/min/mg protein. The ability of this enzyme preparation to convert d^3-iPP to d^2-iPP was demonstrated by two control experiments. The first control consisted of boiled transferase enzyme and active isomerase in the isopentenyltransferase assay using 5'-AMP and ^{14}C-d^3-iPP. In the second control, both enzyme preparations were boiled in a parallel reaction. Results from the Dowex elution profile revealed two peaks of ^{14}C counts corresponding to the d^2 and d^3 forms of iPP from the first control (Fig. 6A). The second control yielded only one radioactive peak, indicating that the d^3 form was present and that isomerization had not occurred (Fig. 6b). These results were confirmed by comparing the elution of authentic ^{14}C-d^3-iPP to the controls.

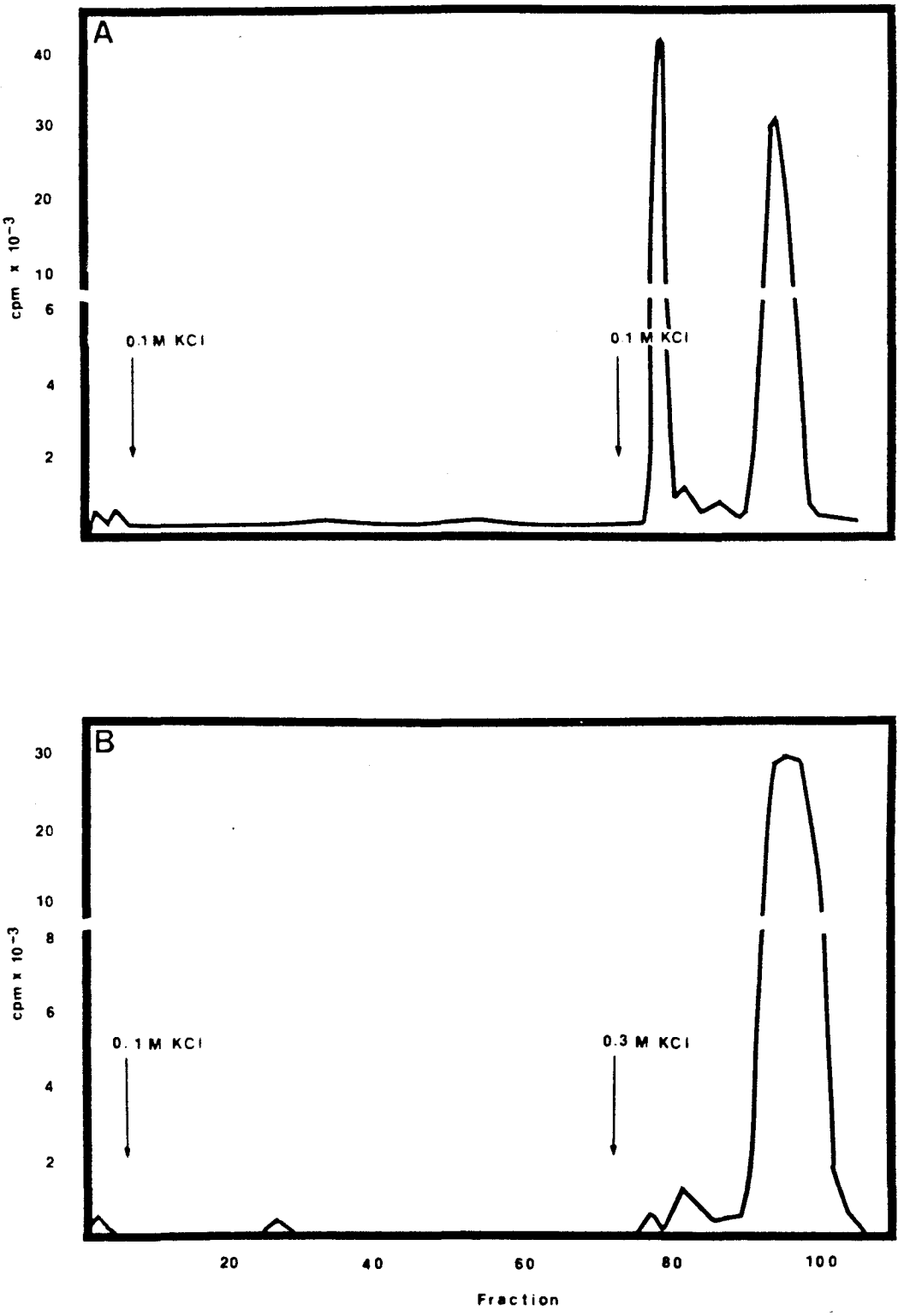
Isopentenyltransferase Assay

Several assay parameters were tested in the assay of d^2-isopentenyltransferase. Since the enzyme had not previously been assayed in yeast, initial parameters were

Figure 6. Dowex Elution Profiles (Control). The reaction mixture was loaded directly onto the column.

A. The reaction mixture contained boiled isopentenyltransferase and active isomerase enzymes. Products eluted correspond to d^2-iPP and d^3-iPP respectively.

B. The reaction mixture contained both boiled isopentenyltransferase and isomerase enzymes. Only one peak corresponding to d^3-iPP was eluted.


A
40
30
20
10
6
4
2
cpm x 10⁻³
0.1 M KCl
0.1 M KCl
B
30
20
10
8
6
4
2
cpm x 10⁻³
0.1 M KCl
0.3 M KCl
20
40
60
80
100
Fraction

modeled after procedures used in slime mold (Taya et al., 1978) and tobacco (Chen & Melitz, 1979; Nishinari & Syono, 1980b). Table 1 summarizes the various assay conditions of each experiment. Three substrates were tested (5'-AMP, adenosine and adenine) for the ability to accept the isopentenyl group from d^2-iPP. Figure 7 illustrates the reactions which were tested.

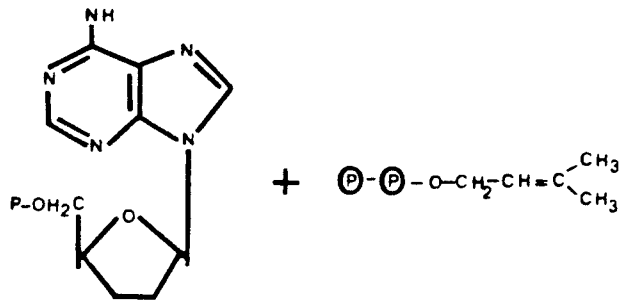
5'-AMP as Substrate

Initially, unlabelled 5'-AMP and ^{14}C-iPP was used in the reaction following the procedure of Chen and Melitz (1979). Enzymatic products could not be identified when this protocol was followed. The next set of experiments were conducted using ^{3}H-labelled 5'-AMP and ^{14}C-iPP. In these cases, a dual-labelled product which could be identified by more than one chromatographic method was anticipated. Experiment 4 compared the assay protocol adapted from the procedures of Chen and Melitz (1979) to that of Taya et al. (1978). Assay conditions modeled according to Chen's procedure resulted in a radioactive profile lacking any dual-labelled peaks. However the procedure similar to Taya et al. yielded two ^{3}H/^{14}C peaks eluting in the first step of the gradient (Fig. 8). In the control run, the second dual-labelled peak disappeared from the Dowex elution profile (Fig. 9). Fractions corresponding to the remaining dual-labelled peak (A2) were combined and one ml (3984 cpm

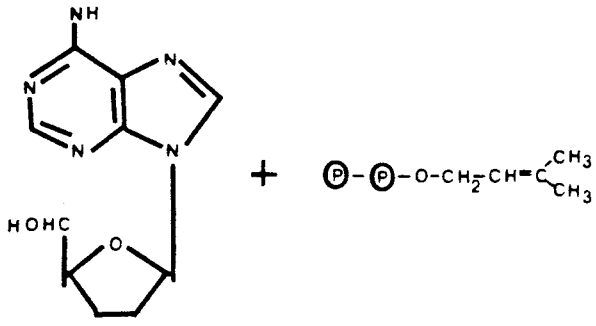
Figure 7. Substrates tested in the isopentenyltransferase assay:

1. 5'AMP + d^2-iPP
2. Adenosine + d^2-iPP
3. Adenine + d^2-iPP

1.



2.



3.

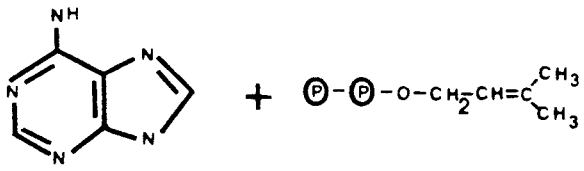


Figure 8. Dowex Elution Profile: ^{3}H-5'-AMP + ^{14}C-iPP (Experiment 4). Two dual-labelled products, A1 and A2, eluted in the first step of the gradient. ^{3}H cpm are represented by the dashed tracing and ^{14}C cpm are represented by the solid tracing. 3.5 ml fractions were collected and 1.0 ml aliquots were taken for liquid scintillation counting.

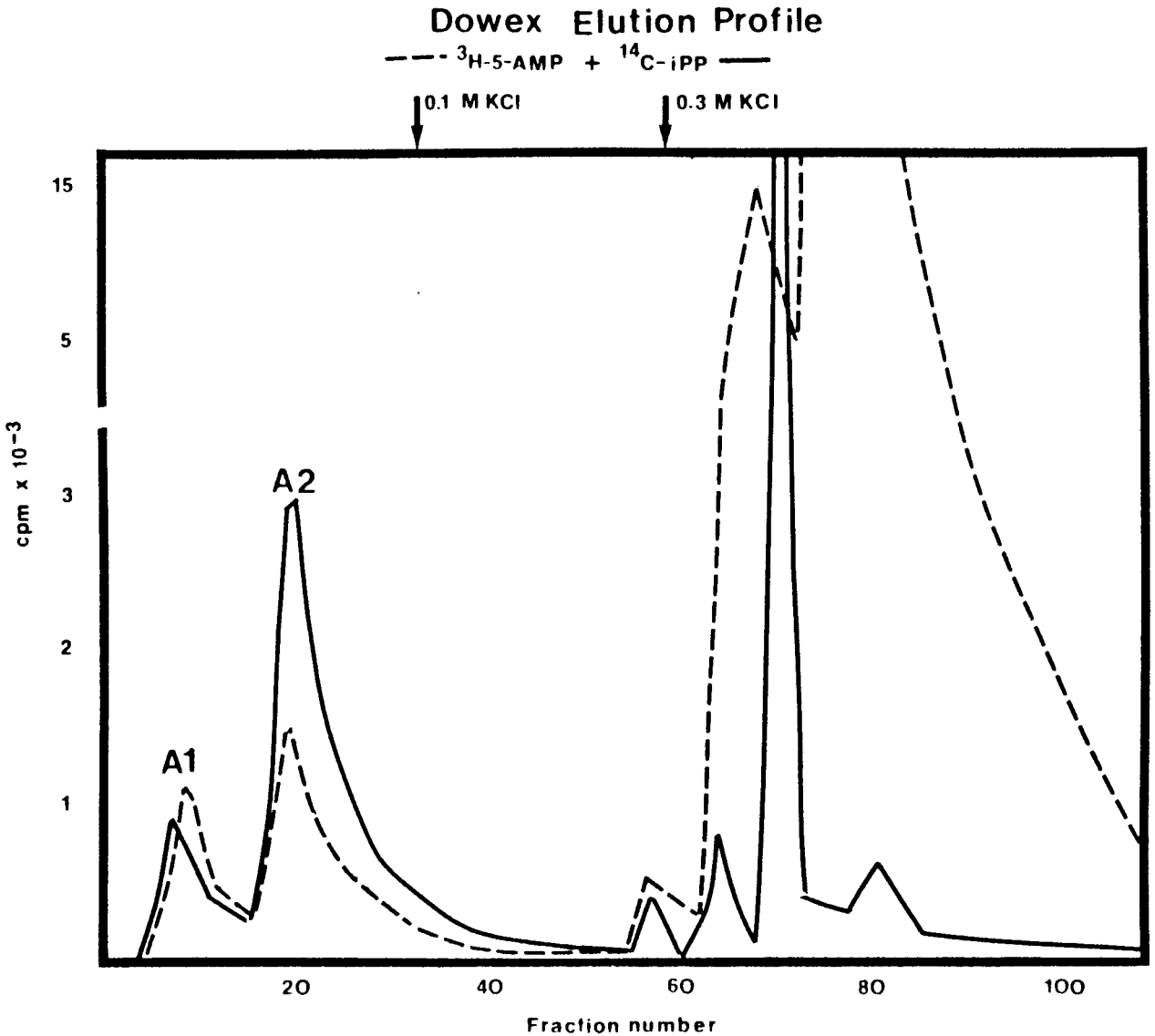
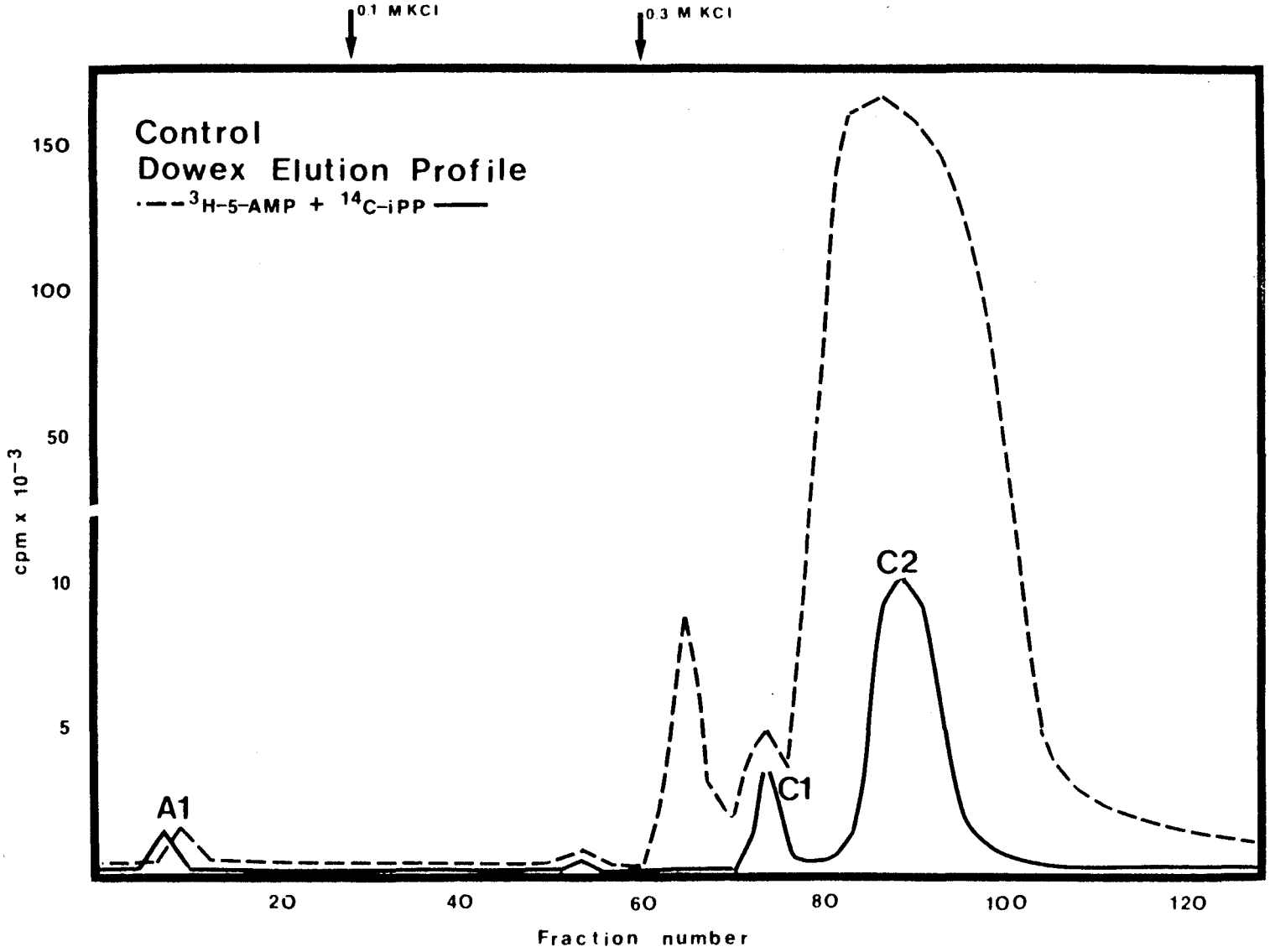

Dowex Elution Profile
^{3}H-5-AMP + ^{14}C-iPP
0.1 M KCl
0.3 M KCl
cpm x 10^{-3}
15
5
3
2
1
A1
A2
20
40
60
80
100
Fraction number

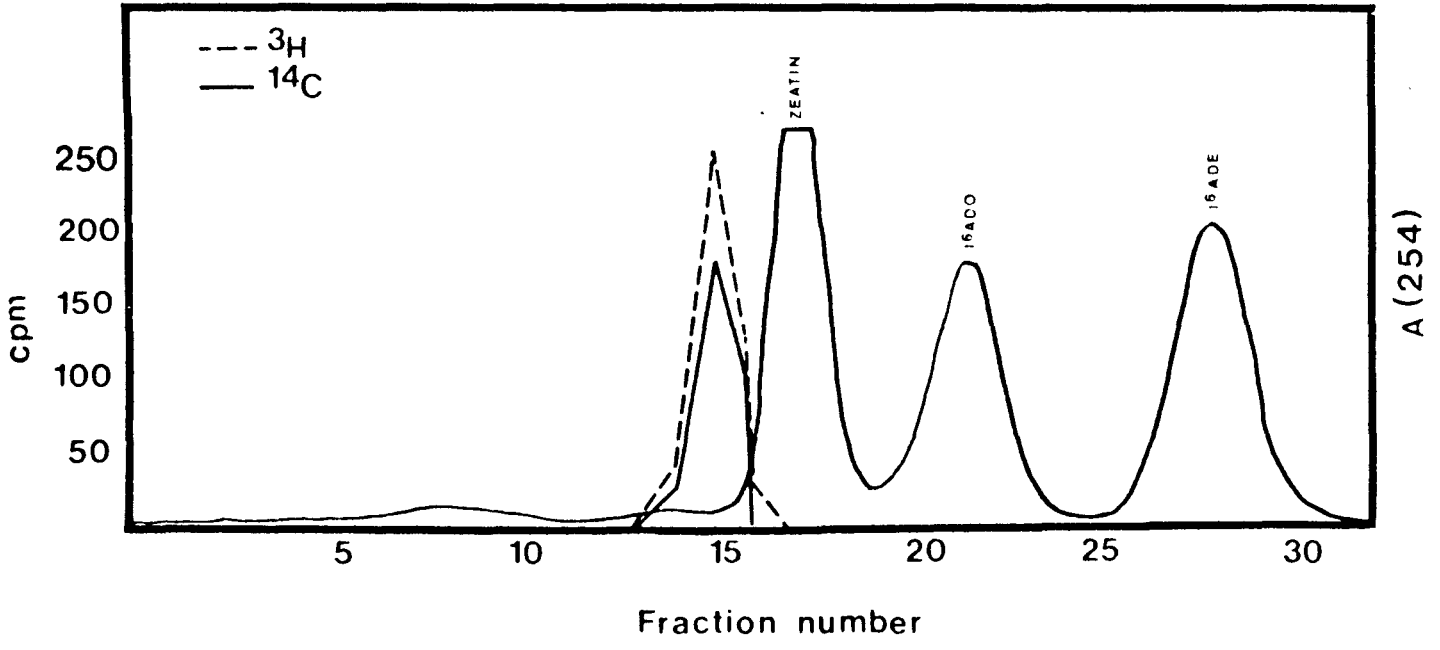
Figure 9. Dowex Elution Profile (Control) ^{3}H-5'-AMP + ^{14}C-iPP (Experiment 4). Boiled isopentenyltransferase and isomerase preparations were used in the reaction. ^{3}H cpm are represented by the dashed line and ^{14}C cpm are represented by the solid line. C1 and C2 correspond to d^{2}-iPP and d^{3}iPP respectively. Peak A2 has disappeared from the elution profile.


0.1 M KCl
0.3 M KCl
Control
Dowex Elution Profile
·——³H-5-AMP + ¹⁴C-iPP——
150
100
50
10
5
cpm x 10⁻³
A1
C1
C2
20
40
60
80
100
120
Fraction number

^{14}C, 2292 cpm ^{3}H) was loaded onto a Sephadex LH20 column. Standards of authentic $I^{6}Ado$, $I^{6}Ade$, and zeatin were co-chromatographed with the sample. Figure 10 illustrates the LH20 elution profile showing a dual-labelled component which elutes just before zeatin. Again the radioactive fractions were combined and concentrated by rotoevaporation for HPLC injection. Due to the volatility of the compound (radioactive counts repeatedly recovered in the trap after rotoevaporation) further analysis on HPLC was not possible. In an attempt to circumvent the need for rotoevaporation, a quicker procedure was adopted for product identification. A Sephadex G-25 column was used as described in Materials and Methods. This column allowed the separation of most proteins from any cytokinin molecules. This step replaced the Dowex anion exchange column thus eliminating the alkaline pH conditions. Since the G-25 eluent did not contain salt, the need for the LH20 column was also eliminated, and direct injection into the HPLC was possible. HPLC elution profiles of control and experimental assays using this protocol clearly indicates a dual-labelled component present only in the experimental run. This product eluted just prior to the $I^{6}Ado$ standard. The majority of the radioactivity in both the experimental (Fig. 11a) and control (Fig. 11b) profiles is associated with fractions containing 5'-AMP and $I^{6}AMP$ standards. Adenine, adenosine, d^{2} and d^{3}-iPP standards are known to chromatograph in this area as well. The same

Figure 10. LH 20 Elution Profile. Peak A2 (3984 cpm ^{14}C; 2292 cpm ^{3}H) obtained from the rection ^{3}H-5'-AMP + ^{14}C-iPP was further chromatographed on Sephadex LH 20. Standards of $I^{6}Ado$, $I^{6}Ade$, and zeatin were co-chromatographed. The eluent was monitored at 254 nm. 4.5 ml fractions were collected, 0.5 ml aliquots were taken for radioactive counting. A single dual-labelled peak was eluted.

Sephadex LH 20 Elution Profile



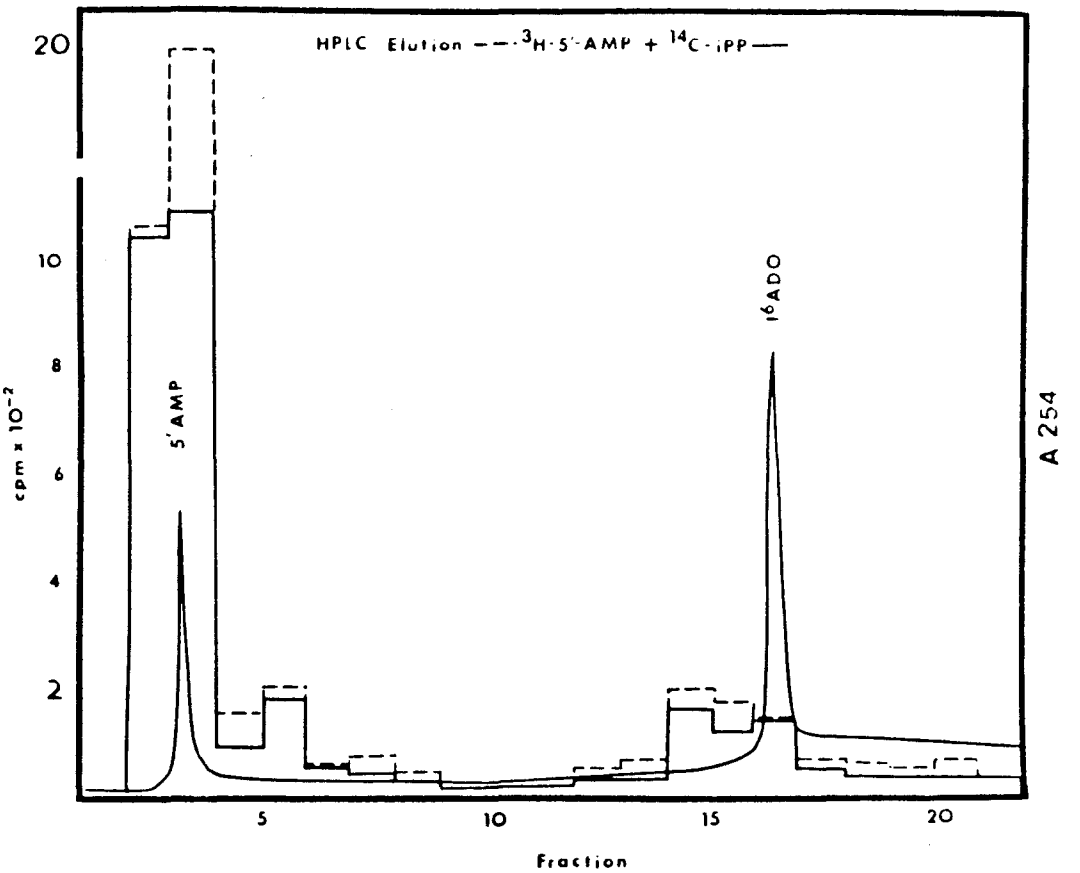


Figure 11a. HPLC Elution Profile: Experimental ^{3}H-5'-AMP + ^{14}C-iPP (Experiment 7). Standards were simultaneously injected with 50 ul of sample (14,261 cpm ^{3}H; 22,092 cpm ^{14}C) and eluted by a 30-70% aqueous methanol gradient. This profile represents products obtained after a 30 minute incubation. 1.0 ml fractions were collected, 0.5 ml aliquots were taken for radioactive counting. Dashed bars indicate ^{3}H counts and solid bars indicate ^{14}C counts.

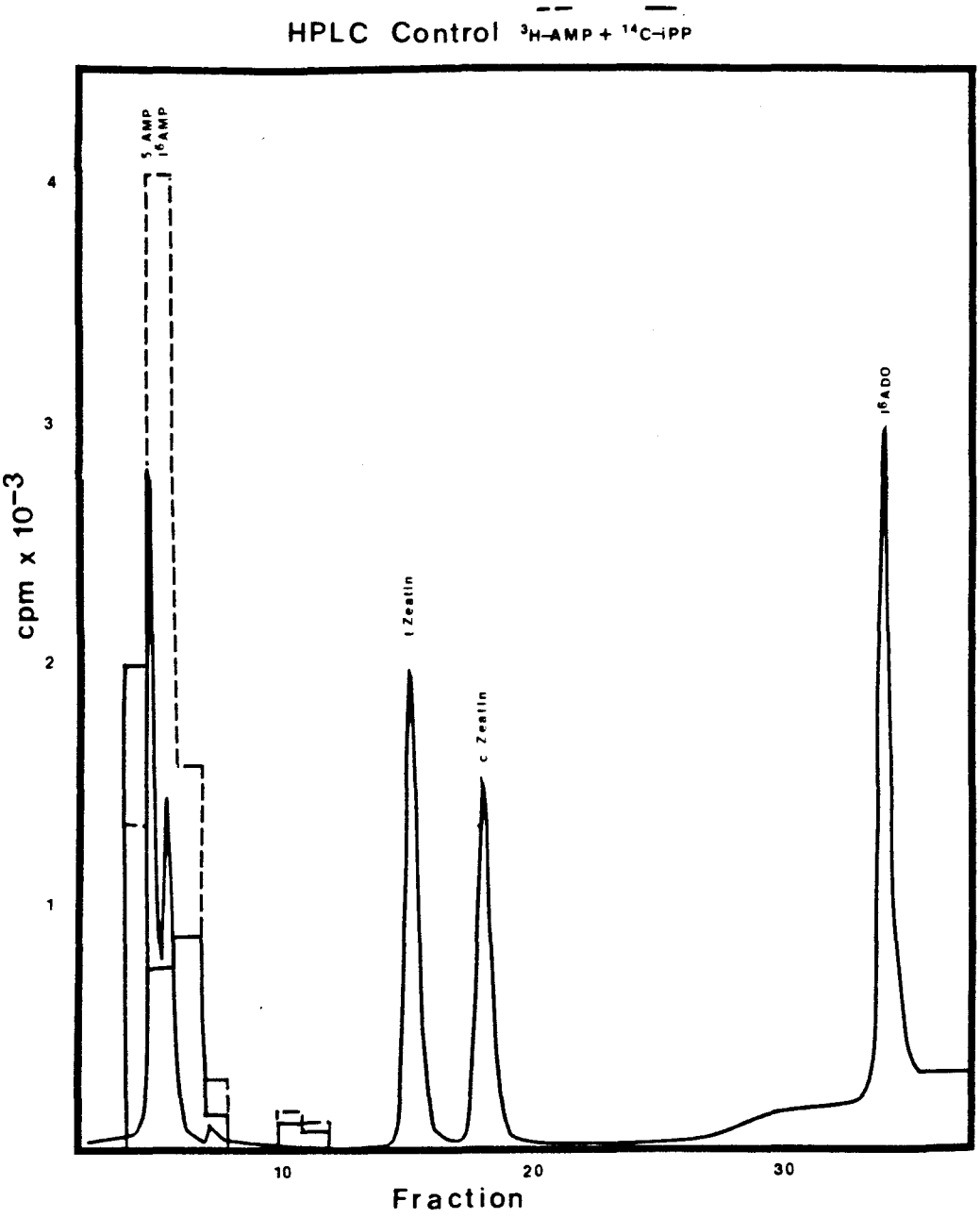


Figure 11b. HPLC Elution Profile: ^{3}H-5'AMP + ^{14}C-iPP. Control-Boiled Enzymes

assay conditions using a 15 minute incubation period resulted in a much smaller dual-labelled peak (results not shown).

Adenosine as Substrate

The ability of adenosine to serve as an acceptor of the isopentenyl group was tested in the next series of assays. A 60 minute incubation was tried first with unlabelled adenosine. Complete assay conditons are listed in Table 1 under Experiment 5. The anion exchange elution profile (Fig. 12) reveals peaks A2 and B1 as possible candidates for an enzyme catalyzed product. Peaks C1 and C2 correspond to d^2 and d^3-iPP respectively. Peaks A2 and B1 were absent in the control run. Peak B1 was then further analyzed on Sephadex LH20 (Fig. 13). Two peaks of ^{14}C counts resulted, L1 and L2. Peak L2 co-eluted with a zeatin standard. Fractions in peak L2 were combined and found not to be volatile during rotoevaporation. Final confirmation of the identity of L1 and L2 by HPLC yielded unexpected results. Both co-eluted with an adenosine standard, which eluted earlier than the zeatin standard (results not shown).

The next experiment made use of ^{3}H-labelled adenosine, and the reaction was also incubated for 60 minutes (Fig. 14). Peak A2 was $^{3}H/^{14}C$ labelled and eluted in the first step of the gradient. An aliquot of this peak (1050 cpm ^{3}H, 1110 cpm ^{14}C) was chromatographed on LH20 and resulted in a dual-labelled peak which eluted between zeatin riboside and

Figure 12. Dowex Elution Profile: Adenosine + ^{14}C-iPP (Experiment 5). The elution profile reveals four peaks of radioactivity: A2, B1, C1, and C2.

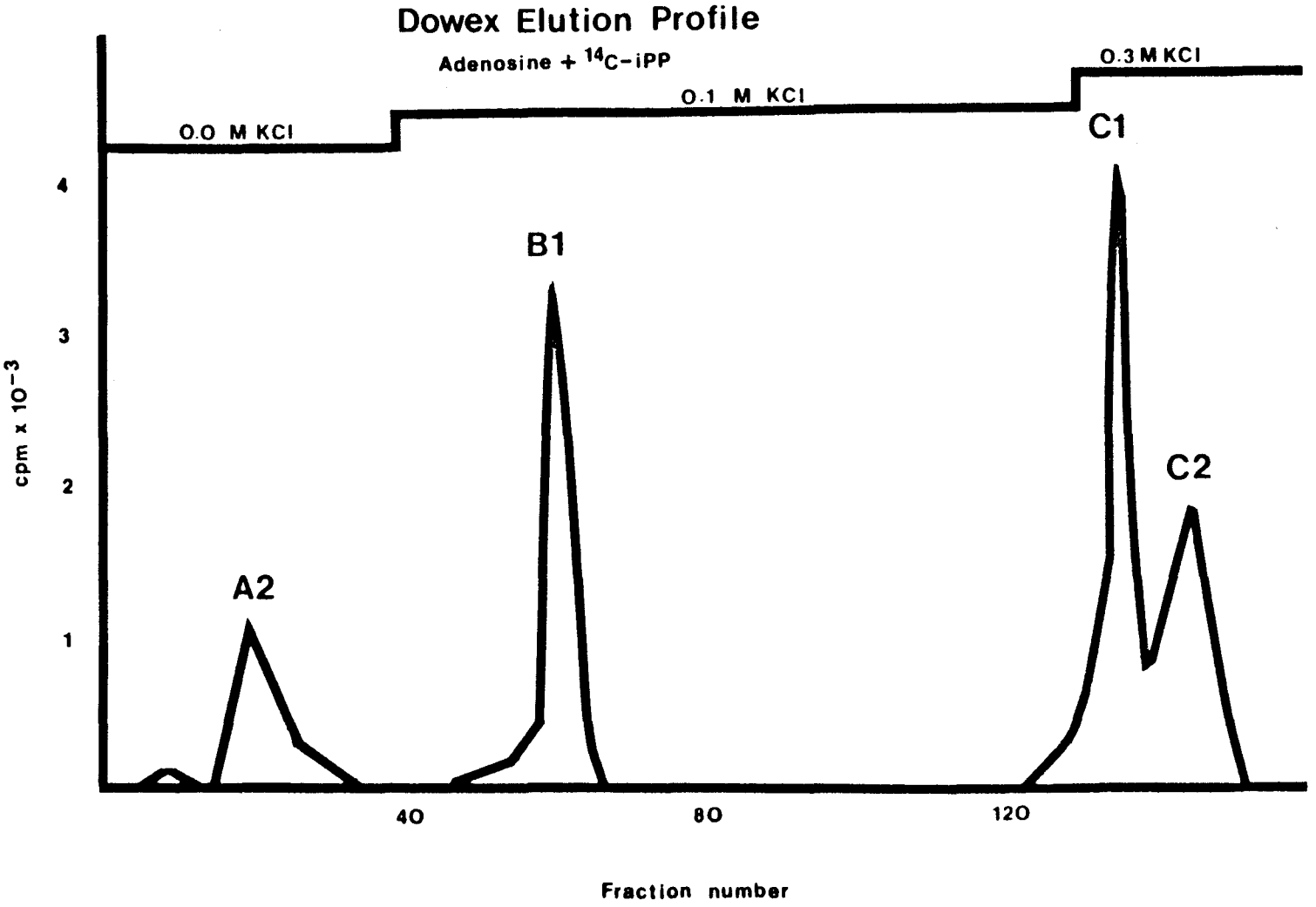

Dowex Elution Profile
Adenosine + ^{14}C-iPP
0.0 M KCl
0.1 M KCl
0.3 M KCl
A2
B1
C1
C2
cpm x 10^{-3}
1
2
3
4
40
80
120
Fraction number

Figure 13. LH 20 Elution Profile. An aliquot of peak B1 (1700 cpm ^{14}C) along with zeatin, zeatin riboside, and I^6Ado standards were loaded onto a Sephadex LH 20 column. Fractions of 4.0 ml were collected and 0.5 ml aliquots were removed for counting. The data points represent ^{14}C counts. Absorbance was monitored at 254 nm.

LH-20 Elution Profile

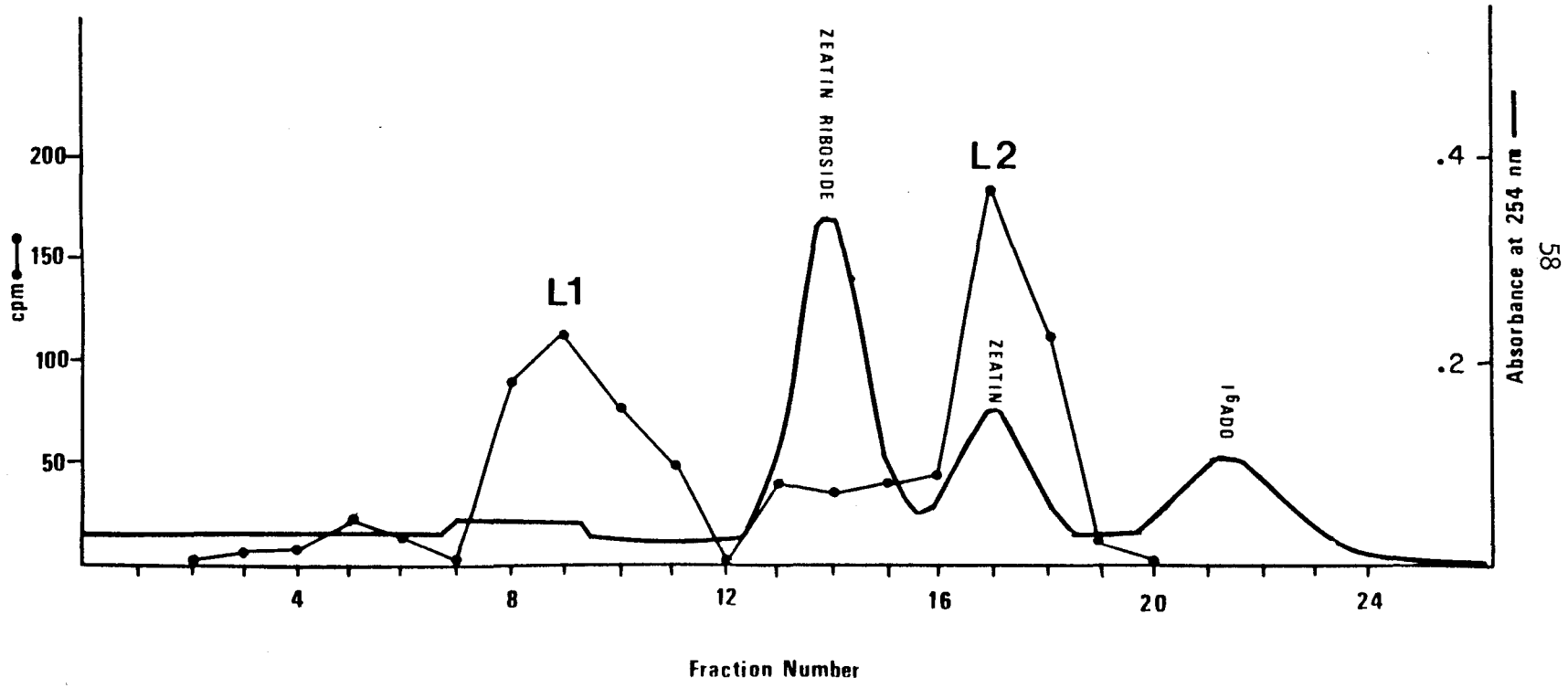


Figure 14. Dowex Elution Profile: ^{3}H-Adenosine + ^{14}C-iPP (Experiment 6). The reaction was incubated for 60 minutes, and loaded onto a Dowex column. The dashed line corresponds to ^{3}H cpm and the solid line corresponds to ^{14}C cpm. Peak A2 is a dual-labelled peak. Peaks C1 and C2 represent d^{2}-iPP and d^{3}-iPP respectively.

zeatin standards (data not shown). The same reaction incubated for 30 minutes also resulted in a dual-labelled peak when run on HPLC. Similarly, this peak did not co-elute with any of the standards on HPLC (Fig. 15).

Adenine as Substrate

Adenine was tried in the reaction mixture as the isopentenyl group acceptor. Initial results showed a ^{14}C peak eluting in the first step of the anion exchange column, which appeared to be identical to A2 (Fig. 16). However due to the low level of radioactive incorporation, further identification of this product was not pursued.

Expression of the Mitochondrial Genome

In an attempt to discern whether the gene for isopentenyltransferase is located on the mitochrondrial genome, enzyme isolation was performed from yeast grown in medium containing glycerol (YPG). The presence of the non-fermentable carbon source, glycerol, evokes expression of the mitochondrial genome. Fewer mitochondria are present in glucose-repressed cells than in fully respiring cells such as when glycerol is present (Stevens, 1981). Wild type yeast was used in this experiment and the enzyme assays were done with ^{14}C-d^3-iPP and ^{3}H-adenosine or ^{3}H-5'-AMP at two time points each. In these experiments an aliquot of the reaction mixture was directly injected into the HPLC. In both cases, the 10 minute incubation resulted in

Figure 15. HPLC Elution Profile: ^{3}H-Adenosine + ^{14}C-iPP (Experiment 7). The reaction was incubated for 30 minutes, and approximately 5000 cpm ^{3}H and 10,000 cpm ^{14}C in 75 ul was injected into the HPLC. The column was eluted with a 30-70% aqueous methanol gradient, fractions were collected directly into scintillation vials.

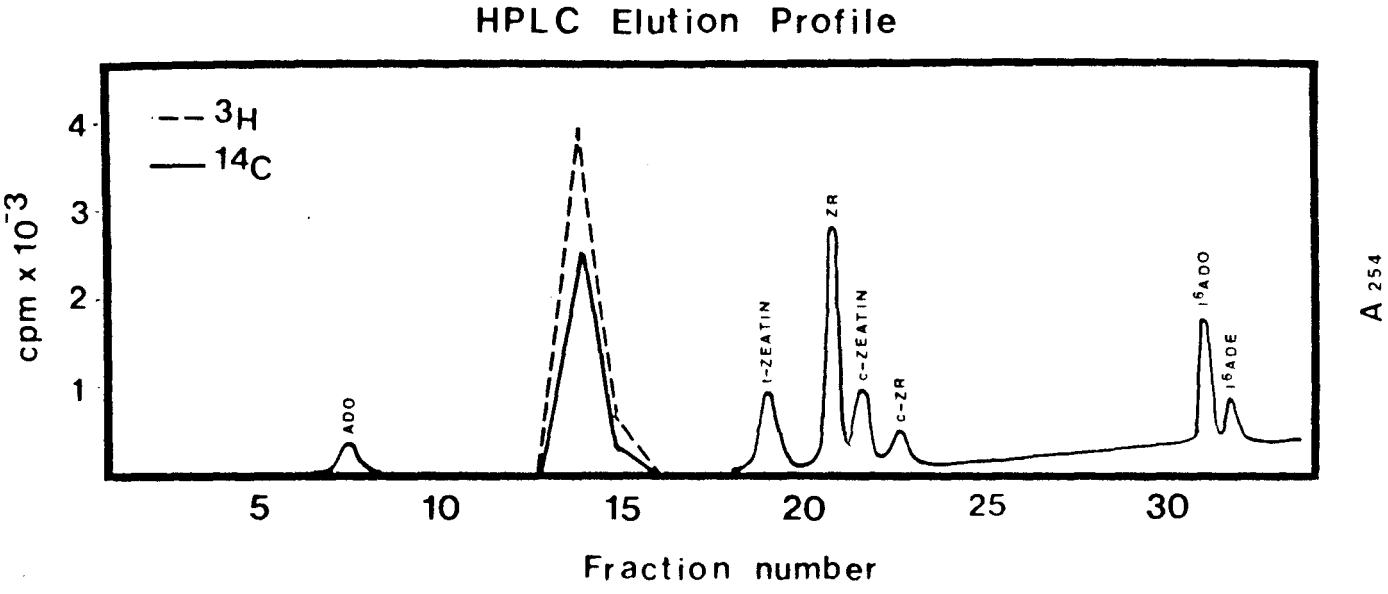
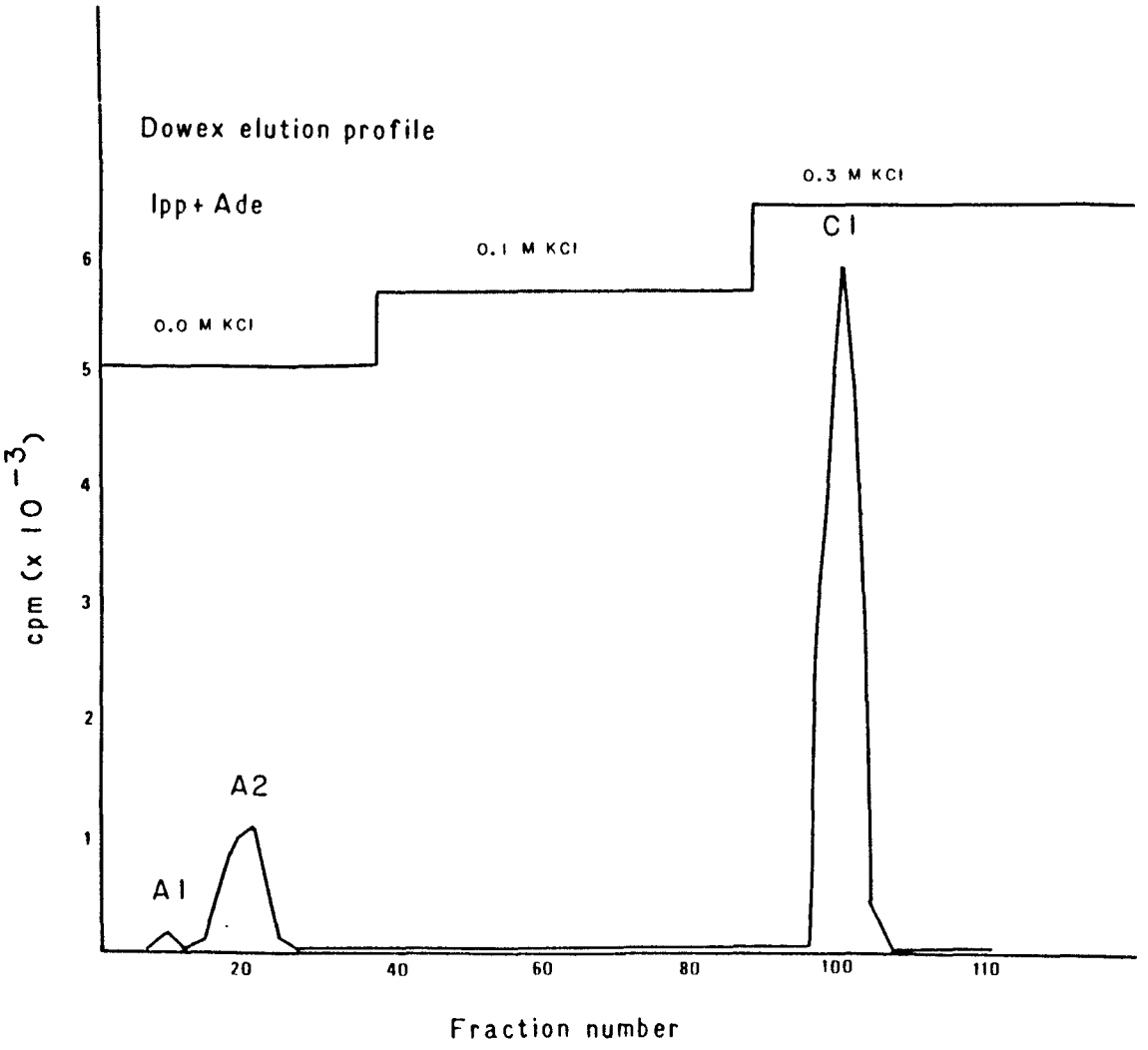

HPLC Elution Profile
--- 3H
— 14C
cpm x 10-3
4
3
2
1
ADO
i-ZEATIN
ZR
c-ZEATIN
c-ZR
i6ADO
i6ADE
5
10
15
20
25
30
Fraction number
A 254

Figure 16. Dowex Elution Profile: Adenine + ^{14}C-iPP (Experiment 5). The reaction was incubated for 60 minutes, and loaded onto a Dowex column.


Dowex elution profile
Ipp+Ade
0.0 M KCl
0.1 M KCl
0.3 M KCl
A1
A2
C1
cpm (x 10^{-3})
Fraction number
1
2
3
4
5
6
20
40
60
80
100
110

^{3}H and ^{14}C counts clustered at the beginning of the elution profile co-eluting with 5'-AMP and $I^{6}AMP$. The 30 minute incubation in both cases (^{14}C-d^{3}-iPP and ^{3}H-adenosine or ^{3}H-5'-AMP) yielded a dual-labelled product with a slightly longer retention time than zeatin riboside (Fig. 17).

Cell Labelling with ^{14}C-Adenine

Wild type yeast cells were grown in the presence of ^{14}C-adenine for two incubation periods, 2.0 hours and 6.0 hours. The labelled cells were harvested, lysed, and the intracellular contents were prepared for HPLC analysis as described in Materials and Methods.

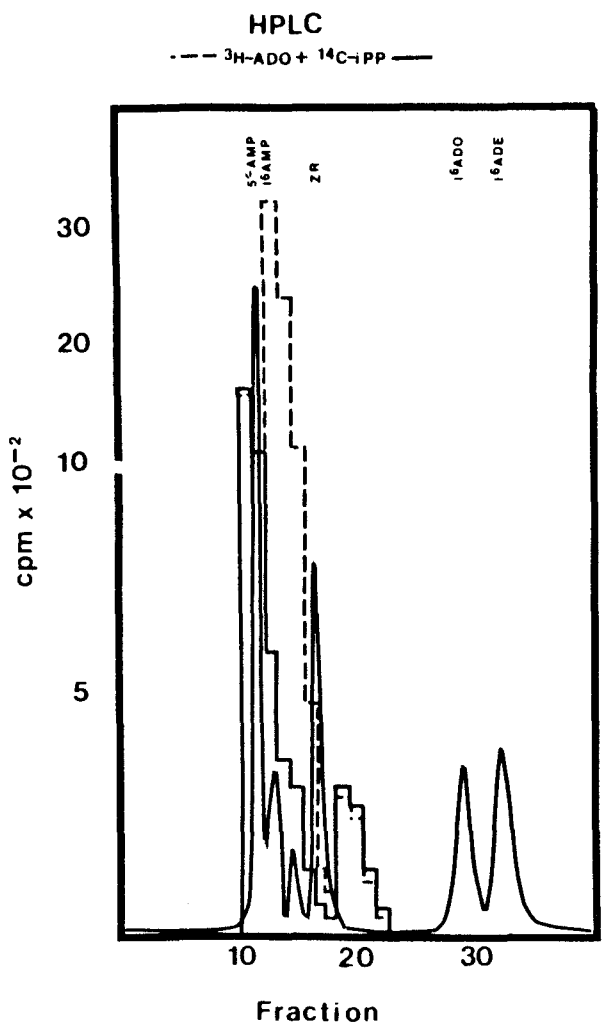
At the 2.0 hour time point, two peaks of radioactivity are apparent (Fig. 18). Peak A eluted prior to any standard, indicating that it was a more polar compound. Peak B eluted just prior to the adenine standard in the vicinity that adenosine would be expected to elute. No radioactivity was found to be associated with any of the later eluting cytokinins (zeatin riboside, $I^{6}Ado$, $I^{6}Ade$).

The 6.0 hour time point resulted in only one peak of radioactivity which co-eluted with adenine.

Figure 17. HPLC Elution Profile: Mitochondrial Expression (Experiemnt 8). Standards and sample (5 ul reaction mixture) were directly injected into the HPLC. The dashed bars represent ^{3}H cpm and the solid bars represent ^{14}C cpm.

A. ^{3}H-Adenosine + ^{14}C-iPP

B. ^{3}H-AMP + ^{14}C-iPP


HPLC
3H-ADO + 14C-iPP
5'AMP
i6AMP
ZR
i6ADO
i6ADE
cpm x 10-2
30
20
10
5
10
20
30
Fraction

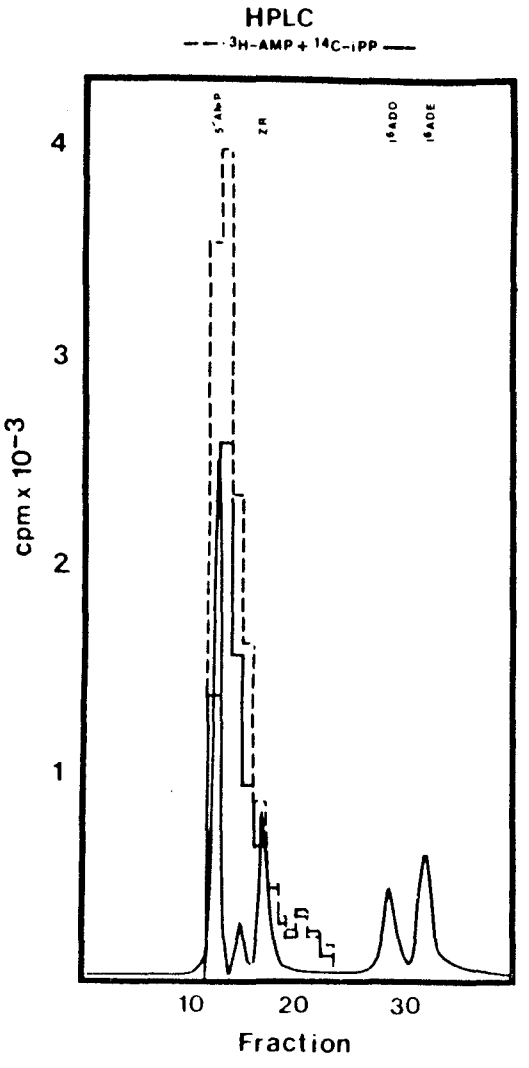
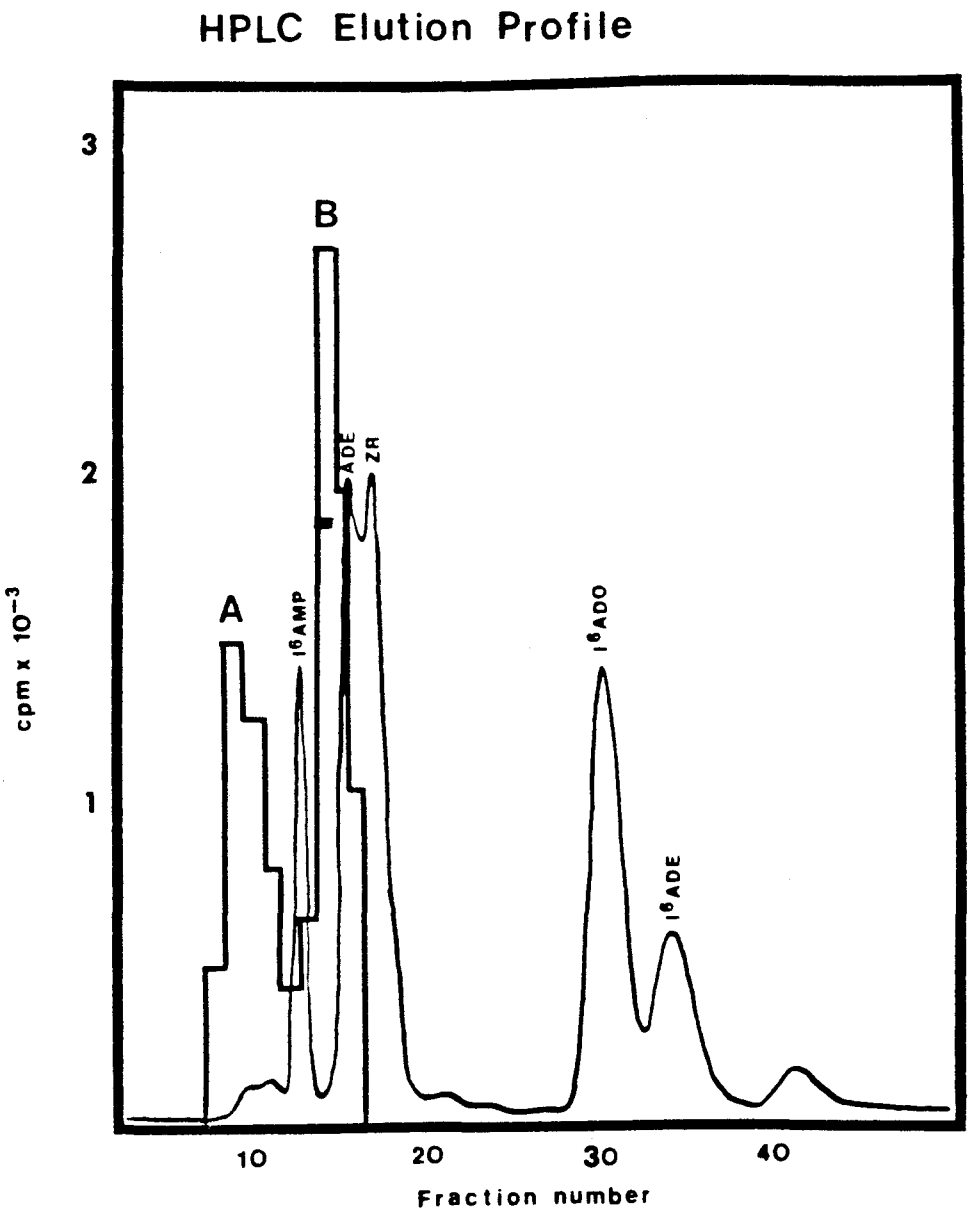

HPLC
3H-AMP + 14C-iPP
5'AMP
ZR
i6ADO
i6ADE
cpm x 10-3
4
3
2
1
10
20
30
Fraction

Figure 18. HPLC Elution Profile: ^{14}C-Adenine labelling. 100 ul of sample and standards were injected into the HPLC. Isocratic elution (55% aqueous methanol) revealed two peaks of radioactivity, A and B.


HPLC Elution Profile
3
2
1
cpm x 10^{-3}
A
B
i^6AMP
ADE
ZR
i^6ADO
i^6ADE
10
20
30
40
Fraction number

CHAPTER V

DISCUSSION

To date, the only non-plant associated systems shown to possess a *de novo* pathway for cytokinin production are slime mold (Taya *et al.* 1978) and the crown-gall inducing bacterium *A. tumefacians* (Barry *et al.*, 1984). In addition, Chen and Melitz (1979) and Nishinari and Syono (1988b) have demonstrated the existence of tRNA-independent pathways in tobacco tissue utilizing an *in vitro* cell-free enzyme assay. Direct evidence for the biosynthesis of cytokinins in yeast has not previously been reported. This study represents the first indication that the yeast, *S. cerevisiae* contains an enzyme system capable of synthesizing products derived from ^{14}C-d^2-iPP and ^{3}H-5'-AMP or ^{3}H-adenosine, *in vitro*. Since this enzyme had not been assayed for in yeast previously, the primary objective of this study was to establish and optimize assay conditions. Therefore it was necessary to utilize a crude enzyme preparation in the development of the assay to ensure detection of activity. The source of the enzyme was the *mod* 5-1 mutant. The advantage in using this mutant was the elimination of substrate (d^2-iPP) competition between the tRNA-dependent transferase and the *de novo* transferase.

Substrate Requirements

Previous studies that have examined substrate requirements for the purified tRNA-independent d^2-iPP-isopentenyltransferase enzyme in tobacco tissue (Chen, 1982) slime mold (Ihara et al., 1984), and A. tumefacians (Barry et al., 1984) have tested the purine adenine, the nucleoside adenosine, and the mono-, di-, and trinucleotides AMP, ADP, and ATP as the acceptor molecules. In view of the structure of isopentenyladenosine and its numerous derivatives (Fig. 1), this choice of acceptor substrates seems logical. Comparing the results of previous studies, it is apparent that the substrate requirements differ for each enzyme (Table 2). It might be expected that plants, slime mold and bacteria would possess distinct forms of the enzyme. This study using yeast concludes that 5'-AMP, adenosine, and possibly adenine are all capable of accepting the isopentenyl group from ^{14}C-d^2-iPP to form a single molecule. Adenine has not positively been shown to be an acceptor molecule since dual-labelled studies were not performed. However, the positive identification of products, cytokinins or otherwise, proved to be elusive.

Substrate: 5'-AMP

^{3}H-5'-AMP and ^{14}C-d^2-iPP were shown to yield a dual-labelled product. The retention of the dual label in three chromatographic systems (Dowex anion exchange, Sephadex

TABLE 2.--Summary of Substrate Requirements for Tobacco, Slime Mold, and A. tumefacians Enzymes

Substrate Substrate	Slime Mold* (in vitro)	Tobacco (in vivo)	Tissue** (in vitro)	A. tumefacians*** (in vitro)
Adenine	-	++	-	-
Adenosine	-	NA	-	-
5'-AMP	++	NA	++	++
ADP	+	NA	NA	NA
ATP	-	NA	NA	NA
Inosine	NA	NA	-	NA
cAMP	NA	NA	_	NA
Guanine	NA	NA	_	NA
GMP	NA	NA	-	NA

* Taya et al. (1978).

**Chen and Melitz (1979); Chen (1982).

***Barry et al. (1984).

NA--Results not available.

LH-20, and RP-HPLC) implies that the product was in fact a single molecular species. The volatility of this product however, leads one to question the identity of this compound since cytokinins are not known to be volatile. If this product is indeed a cytokinin, it is apparently a novel compound. Further structural analysis by mass spectrometry or nuclear magnetic resonance is necessary to confirm its structure. Information about the stoichiometric relationship of the product was obtained by calculating the $^{3}H/^{14}C$ ratio and was found to be in agreement with a 1:1 ratio.

Substrate: Adenosine

When adenosine was used in the reaction, both the unlabelled and tritiated forms of the molecule were capable of reacting to yield products which were not found in the boiled control runs. Unlike the reaction utilizing 5'-AMP, the product was not volatilized by rotoevaporation. The stable nature of the compound and the fact that product remained dual-labelled in all of the chromatographic systems was encouraging. However similar to the results using 5'-AMP, the identification of product proved to be difficult because it did not co-elute with any of several commercially available standards.

There was a discrepancy between products obtained using cold adenosine and ^{3}H-adenosine even though all other parameters were comparable. The anion exchange column from

the reaction using unlabelled adenosine resulted in two peaks of radioactivity, A2 (eluted in 0.0 M KCl) and B1 (eluted in 0.1 M KCl) which were both absent in the control. When ^{3}H-adenosine was used, the anion exchange results showed only one dual-labelled peak, A2, which was not found in the control. The absence of B1 in this reaction is puzzling. B1 was shown to be comprised of two components, L1 and L2 which were resolved on Sephadex LH-20. L2 co-eluted with authentic zeatin on LH-20, however upon further analysis on HPLC, the compound eluted much earlier than zeatin, indicative of a somewhat more polar structure than zeatin. This behavior is suggestive of chemical alteration. It is possible that the "zeatin-like" product may have decomposed yielding an altered structure.

Peak A2 which was obtained using ^{3}H-adenosine was intermediate in hydrophobicity between zeatin and zeatin riboside as inferred from Sephadex LH-20 chromatography. Separate experiments at three incubation periods (15, 30, and 60 minutes) revealed a dual-labelled compound on the HPLC, which could not be identified. More rigorous structural analysis as mentioned previously is indicated.

Substrate: Adenine

It appears that adenine can participate in the reaction to yield an enzymatic product. Further experimentation using adenine was not pursued due to the initial results

which showed minimal radioactive incorporation in peak A2 when compared to other substrates used.

In Vivo ^{14}C-Adenine Labelling

Although adenine, adenosine, and 5'-AMP seem to be logical substrates, it is also possible that these molecules may not be specific substrates *in vivo*. Experiments attempting to label cytokinins *in vivo* by growing yeast in the presence of ^{14}C-adenine resulted in no incorporation into cytokinins. In referring to the purine biosynthetic pathway (Fig. 3), it can be seen that there are limited candidates capable of accepting the isopentenyl group. Only adenine, adenosine, and AMP possess nitrogen in position 9 on the purine ring, inosine and hypoxanthine do not. Chen (1982), tested inosine and found it was not a substrate for the enzyme. This study represents the only attempt which tested a purine precursor. Since yeast cells will not take up many compounds (Adenosine, 5'-AMP, Inosine) it would be necessary that these studies be performed *in vitro*. Another explanation for the lack of ^{14}C-adenine incorporation into cytokinins may be found in recent evidence which links mevalonate derived isoprenoids with specific cell proteins (Schmidt *et al.*, 1984). I^{6}Ado is cited among others as a possible candidate in this association. If cytokinins are indeed linked to protein *in vivo*, ^{14}C-adenine labelling would result in incorporation into a protein fraction instead.

Incorporation of label into free I^6Ado might be transient and virtually undetectable.

In addition, it is possible that endogenous levels of cytokinins may fluctuate with the cell cycle. Nishinari and Syono (1980a) introduced the first evidence in support of this showing a 5 to 10 fold increase of endogenous cytokinins which paralleled mitosis in partially synchronized tobacco cells.

Optimization of Assay Parameters

In developing this assay, the only indication of enzymatic activity was in the identification of products which were absent in the control. Initial experiments were adapted from the procedure of Chen and Melitz (1979). Using their buffer system (7.0 mM Tris-HCl, pH 7.0; 20 mM $MgCl_2$; 35 mM $MnCl_2$; 3.5 mM 2-mercaptoethanol; 7.0 mM KF) at 37 C, virtually no product formation was observed. Subsequently the assay system of Taya et al. (1978) was tested (20 mM Tris-HCl, pH 7.6; 5.0 mM magnesium acetate) incubated at 25 C, and adopted for use in all succeeding experiments. Different incubation periods were used in the hope of characterizing the biosynthetic pathway. However due to the difficulty in product identification, this information was not obtainable from the results. In terms of the amount of product formed, thirty minutes to one hour incubation was optimum.

In order to determine whether the gene for d^2-iPP-isopentenyltransferase might be located on the mitochondrial genome, and therefore would require mitochondrial expression, the yeast cells were grown in YPG medium which contains glycerol as the carbon source. Results of the assays when the enzyme was prepared from cells grown in YPG medium did not appear to be different than those when enzyme was prepared from cells grown in YPD medium. This suggests the enzyme(s) responsible for the synthesis of the products observed were probably not mitochondrial.

Summary

The mod 5-1 mutant of Saccharomyces cerevisiae has been shown to possess an enzyme system responsible for the apparent isopentenylation of 5'-AMP, adenosine, and adenine using d^2-iPP as the donor molecule. The failure of these products to co-elute with any authentic cytokinin implies that these products may be novel compounds not yet identified as cytokinins. Several investigators have described metabolic enzymes which interconvert these molecules to different forms. These enzymes include 5'-nucleotidases which dephosphorylate cytokinin nucleotides (Chen & Kristopeit, 1981a), adenosine nucleosidases which are responsible for the deribosylation of cytokinin nucleosides (Chen & Kristopeit, 1981b), adenine phosphoribosyltransferases which converts cytokinin bases to their corresponding nucleotides (Chen et

al., 1982), and mixed function oxidases which hydroxylate I^6Ade and I^6Ado to zeatin and zeatin riboside respectively (Chen, 1982). It is possible that yeast may contain other metabolic enzymes, thus generating alternate structures.

Obviously more structural analysis is needed to identify these products, in addition to a cytokinin bioassay to determine whether these molecules have cytokinin activity in plants. The importance of the biosynthesis of these molecules in yeast may be significant in light of their proposed involvement in DNA replication (Quesney-Huneeus et al., 1980), their role as anti-tumor agents (Trewyn & Kerr, 1979; Tritsch, 1973) and their immunosuppressive effects (Hacker & Feldbush, 1969). Yeast represent an excellent organism in which to study the mechanism of these effects.

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APPROVAL SHEET

The thesis submitted by Debra A. Tonetti has been read and approved by the following committee:

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

Dr. Rosemary R. Grady
Assistant Professor, Biology, Loyola

Dr. Mary Druse Manteuffel
Associate Professor, Biochemistry, Loyola

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 16, 1985
Date

Howard M Laten
Director's Signature