METABOLIC STUDIES OF N^6-(Δ^2- ISOPENTENYL)ADENOSINE IN NORMAL AND AUTONOMOUS TISSUES OF *Nicotiana tabacum L.*

By

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ABSTRACT

The nucleoside, N^6-(Δ^2-isopentenyl)adenosine (i^6Ado) stimulates growth and differentiation of lines of tobacco callous tissue grown in culture. The nucleoside is extensively metabolized by the plant tissue. The hypothesis has been advanced that the expression of growth and differentiation is due to the interaction of some or all the metabolites of i^6Ado rather than due to i^6Ado alone or one of its metabolites.

To study this hypothesis, the metabolism of i^6Ado was followed in two lines of tobacco callous tissue. One line, KX, required the presence of i^6Ado and auxin for growth, the second line, an autonomous line, 0-1, required neither hormone for growth. Both lines metabolized [8-^{14}C]i^6Ado, yielding a mixture of 12 metabolites, 9 of which were identified. Quantitatively the two patterns of metabolism differed. The principle product in the KX tissue was ribosylzeatin, whereas in the 0-1 tissue it was N^6-(Δ^2-isopentenyl)adenosine.

The effect of other plant hormones, auxin and abscisic acid on the metabolism of i^6Ado was studied. Auxin had no effect on i^6Ado metabolism in 0-1 tissue, but in KX tissue it depressed the rate of uptake. Abscisic acid caused a change in ratios of i^6Ado metabolites in KX tissue, but not in the autonomous tissue.

These data do not prove or disprove the hypothesis mentioned above, they do indicate, however, that a varied pattern of metabolic products of i^6Ado are formed in normal tobacco callous tissue.

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ABBREVIATIONS USED

ABA	-	Abscisic acid
Ade	-	Adenine
Ado	-	Adenosine
BuOH	-	Butyl alcohol
DMF	-	Dimethylformamide
EtOAc	-	Ethyl acetate
HOAc	-	Acetic acid
HOFc	-	Formic acid
i^6Ade	-	N^6-(Δ^2-isopentenyl)adenine
i^6Ado	-	N^6-(Δ^2-isopentenyl)adenosine
i^6Ado-5'-P or i^6Amp	-	N^6-(Δ^2-isopentenyl)adenosine monophosphate
IU	-	International units
NAA	-	Napthaleneacetic acid
PrOH	-	Propyl alcohol
RZ	-	Ribosyl zeatin
RZ-5'-P	-	Ribosyl zeatin-5'-monophosphate
TLC	-	Thin-layer chromatography
2ms i^6Ado	-	N^6-(Δ^2-isopentenyl)2-methylthio-adenosine
Z	-	Zeatin

I. INTRODUCTION

One of the hypermodified nucleosides, N^6-(Δ^2-isopentenyl)adenosine (i^6Ado), has been detected in certain tRNA species of all kinds of living organisms. This modified nucleoside has been shown to be located at the position adjacent to the 3'-end of the anticodon loop. In some cases, however, the parent nucleoside, N^6-(Δ^2-isopentenyl)adenosine and its structurally related compounds can be obtained in unbound forms, i.e. they are found not as part of tRNA molecules. The free form of i^6Ado and its derivatives are found only in higher plants.

This modified nucleoside has been shown to evoke a variety of physiological responses in different biological systems. In animal tissues, it stimulates the release of circulating white blood cell precursors in rats (Suk *et al.*, 1970) and the development of isolated chick embryonic ganglion cells (Hall *et al.*, 1972). It also inhibits the growth of leukemic cells *in vivo* (Suk *et al.*, 1970) and *in vitro* (Rathbone and Hall, 1972) and the mitosis of phytohemagglutinin stimulated human lymphocytes (Gallo *et al.*, 1969). In plant systems, i^6Ado exhibits cytokinin activity and is considered to be one of the plant hormones. Application of cytokinins to an intact plant releases apical dormonancy and stimulates lateral bud formation. The addition of this compound to detached leaves retards senescence, presumably by inhibiting protein degradation or the synthesis of proteolytic enzymes. It also exerts growth and differentiation of tobacco callus in co-ordination with

another plant hormone, auxin.

In all these systems, although a large amount of data has been accumulated concerning the biological activity of i^6Ado, the molecular basis for these phenomena has not been explained.

In order to understand the mechanism of cytokinin action in plant systems, numerous approaches have been undertaken. One of the questions asked is whether or not cytokinin compounds are incorporated into tRNA. Because i^6Ado occurs as a component of specific tRNA molecules, it was suggested that the cytokinin action involves the function of tRNA. This hypothesis was based on the findings of Fox (1964), who showed that radioactive labelled benzyladenine, a synthetic cytokinin, was incorporated into sRNA fractions of tobacco callus nucleic acids. Similar observations were subsequently reported by Fox (1965, 1966), Fox and Chen (1967, 1968) and Srivastava (1966). These workers suggested that cytokinin action occurred due to its incorporation into tRNA.

However, when similar experiments were repeated by Kende and Tavares (1968), using 6-benzylamino-9-methylpurine, labelled in the methylene carbon, they were unable to detect incorporation of radioactive materials into the reported fractions of sRNA (tRNA) by Fox and Chen (1967, 1968). Moreover, this compound supports the growth of soybean callus tissues. These findings show that cytokinin activity is not due to the incorporation of the synthetic analog into the nucleic acid fractions.

The initial experiments of Fox and Chen were re-examined by Dyson, who was working in Fox's lab at that time. In his thorough study, using a similar MAK column, Dyson demonstrated, as reported in his

thesis, that the level of benzyladenine incorporation was considerably less than that reported by Fox and Chen. Moreover, this low level of incorporation turned out to be in higher molecular weight RNA rather than the tRNA. Additional evidence against the incorporation of the synthetic cytokinin into tRNA was also reported by Richmond *et al.*, (1970) and Benzemer-Sydrary and Veldstra (1971). Elliot and Murray (1972) demonstrated that there was no significant incorporation of benzyl-labelled 6-benzyladenine. The small amount of radioactivity found in tRNA thus could have been due to a transbenzylation reaction.

Biosynthesis of i^6Ado in tRNA occurs by the attachment of an isopentenyl side chain to the appropriate Ado residue of the tRNA. It has been shown that mevalonic acid (Peterkofsky, 1968; Chen and Hall, 1969) and acetate (Fittler *et al.*, 1968a) is the source of Δ^2-isopentenyl pyrophosphate, which is incorporated specifically into the Ado residue located adjacent to the 3'-end of the anticodon, in the preformed tRNA molecule (Kline *et al.*, 1969). The incorporation of mevalonic acid into a specific Ado residue of the tRNA was not depressed by the addition of i^6Ado which was included in the culture medium (Chen and Hall, 1969). These data suggest that i^6Ado *per se* could not be incorporated into the preformed tRNA, because if it were, it should decrease the degree of incorporation of the mevalonic acid into the tRNA molecule.

The enzyme, which catalyzes the transfer of the Δ^2-isopentenyl group to the specific Ado residue in the recipient tRNA molecule, *in vitro*, was isolated from yeast and rat liver (Fittler *et al.*,1968b) and tobacco pith (Chen and Hall, 1969). The enzyme extracted from tobacco pith utilizes Δ^2-isopentenyl pyrophosphate only and not the Δ^3-isomer; whereas

the enzyme extracts obtained from rat liver or yeast catalyze the incorporation of not only the Δ^2-isopentenyl group but also mevalonic acid and the Δ^3-isomer into the preformed tRNA. It was also found that for the rat liver or yeast enzyme preparation, treatment of tRNA with $KMnO_4$ enhances its capacity to accept the isopentenyl side chain. In the case of the tobacco enzyme the Δ^2-isopentenyl side chain can be incorporated only into the $KMnO_4$-treated tRNA *in vitro*. The $KMnO_4$ oxidation is a specific reaction which cleaves the N^6-isopentenyl side chain from the i^6Ado residue of the tRNA. The reaction condition is so mild that it does not affect the rest of the macromolecule (Hall, 1970). Therefore, it is quite certain that the i^6Ado residue in tRNA is synthesized by the attachment of a N^6-Δ^2-isopentenyl group to the highly specific Ado residue only after the macromolecule is formed.

The presence of the cytokinin at a strategic position in the anticodon loop is a significant feature of the tRNA macromolecule. In an elegant experiment, Gefter and Russell (1969) showed that the translational capacity of the three molecular species of suppressor tyrosine tRNA obtained from mutants of *E.coli* were significantly different depending on the presence and nature of the modified nucleosides at the position adjacent to the 3'-end of the anticodon loop. The tRNA containing N^6-(Δ^2-isopentenyl)2-methylthioadenosine, had the highest amino acid transfer activity of the three tRNAs; the one that contained i^6Ado showed intermediate activity whereas the tRNA containing unmodified adenosine had no translational capacity under the same experimental conditions. Fittler, Kline and Hall (1968a) have also demonstrated that the treatment of tRNA with aqueous iodine, another specific reaction for the N^6-(Δ^2-

isopentenyl) side chain, resulted in loss of ability of tRNA containing i^6Ado to bind to ribosomes in the presence of appropriate messenger RNA. However, the ability to accept amino acids was not impaired.

It is clear, therefore, that the presence of i^6Ado in certain tRNA molecules has functional significance in its own right. The strategic position in the anticodon loop and the highly reactive allylic double bond in the side chain apparently help to maintain the integrity of the complementary interaction of the codon-anticodon pair. The correct conformation of the tRNA molecule could be distorted if this modified nucleoside is deleted or modified which could seriously affect not only translational capacity but also the fidelity of translation of the tRNA molecule.

Although the presence of i^6Ado in specific tRNA molecules is functionally significant, it is not clear if this fact and cytokinin activity in plants are related. The available evidence indicates that the cytokinin activity is due to the free form of the modified nucleoside rather than its presence in tRNA (Hall, 1973). However, it does not mean that free cytokinins are not related to the form bound in the tRNA molecule. The degradation of tRNA could be a potential source of free cytokinins in the cells. It has already been suggested that the rate of release of i^6Ado from tRNA and the rate of its metabolism could serve as a balancing mechanism for maintaining the proper intracellular level of this compound in tissues (Chen and Hall, 1969).

The degradation of tRNA, therefore, seems to be one of the sources of the modified nucleoside. The biosynthesis of the free i^6Ado from its immediate precursor, Ado, however, could still occur independent of tRNA.

Recently, Hall (unpublished results) has shown that arabinosyl-Ade, an analog of Ado, was converted into arabinosyl-i^6Ade in an autonomous strain of tobacco callus. It is generally accepted that the arabinosyl analog cannot be incorporated into any form of nucleic acid (Cohen, 1966). This indicates that conversion of arabinosyl-i^6Ade from its immediate precursor arabinosyl-Ade takes place by the direct attachment of a N^6-isopentenyl side chain to the free form of the analog. In other words, the enzyme responsible for the attachment of the Δ^2-isopentenyl group to the appropriate adenosine acceptor could operate at both the macromolecular and the nucleoside level.

This assumption was supported by Einest and Skoog (1973) who showed that i^6Ade and zeatin were synthesized from their immediate precursor [8-^{14}C] Ade in the autonomous tobacco callus but not in a cytokinin dependent strain, Wisconsin No. 38. In addition it has been reported that in the 0 to 1 mm section of pea root tips there is approximately 27 times as much free cytokinin as that present in tRNA (Short and Torrey, 1972). Beulelmann (1973) has also shown that [8-^{14}C] Ade is the direct precursor of i^6Ade in the callus cells derived from the sporogon of the hybrid *Fumaria hygrometrica* x *Physscomitrium piriforme*. The possibility of the synthesis of cytokinin occurring via tRNA breakdown is not feasible because it has been shown that the half-life of tRNA in plant tissue is at least three days (Klemen and Klambt, 1974), and the above conversion occurred in less than three days. These data indicate that there is an alternative biosynthetic pathway of cytokinin other than from tRNA degradation.

Senescence and Cytokinins

In the senescencing process of detached leaves, the mode of cytokinin action is better understood. Chibnall (1939) was the first to observe that detached leaves underwent rapid aging with a loss of protein and chlorophyll. The process was prevented if adventitious roots developed in the petioles. Chibnall, therefore, proposed that a hormone originating in the roots was responsible for a balanced protein metabolism in the leaves and the deficiency of such a compound could lead to senescence Almost 20 years later, Richmond and Lang (1957) succeeded in delaying senescence of detached cocklebur leaves by treating with kinetin (6-fur-furylaminopurine), a synthetic cytokinin which had been discovered at that time. This result was subsequently confirmed by many investigators using a variety of different plants (Osborne and McCalla, 1961; see also references cited in Hall, 1973). Richmond and Lang (1957) also noticed that the protein level in kinetin-treated detached leaves declined more slowly than in the untreated controls. A similar effect of kinetin on RNA level was observed by Osborne (1962), who also found that the incorporation of a labelled amino acid into protein, and of [$^{32}P_i$] or radioactive orotic acid into RNA, was enhanced by treating detached leaves with kinetin.

An attempt has been made to measure the effect of cytokinins on the degradation of leaf proteins. Corn leaves were incubated for 4.5 hours with [^{14}C]-labelled leucine, the incubation mixture was chased by a high concentration of unlabelled leucine in the presence and absence of benzyl-adenine. It was found that the percent loss of both total protein content and radioactivity were higher in the absence of cytokinin (Tavares and

Kende, 1970). Shiboaka and Thimann (1970) have also shown that [^{14}C]-leucine incorporation into protein was not increased in the presence of kinetin, although this compound prevented protein breakdown of the detached leaves of *Pisum sativum.* In addition, it was found that the increase occurred in the activity of proteases (Shiboaka and Thimann, 1970) and Ribonuclease I (Udvardy *et al.*, 1969) within a few hours after the leaves were detached. However, the activity of proteases (Anderson and Rowan, 1966; Beevers, 1968) and RNase (Srivastava, 1968; Sodek and Wright, 1969) was found to be lower if the detached leaves were first treated with cytokinin.

Therefore, it seems to be clear that in retarding senescence cytokinin prevents protein and possibly RNA degradation, perhaps through inhibition of the synthesis of proteases and RNases. It has also been suggested that the synthesis of the degradative enzymes may be retarded by the synthesis of the mRNA responsible for their production.

Martin and Thimann (1972) have demonstrated that cycloheximide, a protein synthesis inhibitor, strikingly prevented senescence of oat leaves and at the same time prevented the incorporation of leucine into protein. It has also been observed that when barley leaves were kept in the dark, yellowing was significantly decreased not only by cytokinins but also by chloramphenicol, puromycin and tetracycline, antibiotics that inhibit directly or indirectly the synthesis of proteins (Hall, 1973).

Although the hypothesis that cytokinin delays senescence by inhibiting protein and RNA degradation, rather than stimulating synthetic activity of macromolecules is generally accepted, it is not known whether cytokinin inhibits the synthesis of the degradative enzymes or their

release from some lysosome-like organelles or through both processes.

The effect of plant hormones on nucleic acid metabolism has been the subject of a great number of investigations. Much of this work has undoubtedly been based on the hypothesis that hormones act by regulating replication or transcription of DNA. The evidence, so far accumulated, indicates that cytokinin activity seems to be independent or at least not directly related to the synthesis of DNA (Schaeffer and Sharpe, 1969; Fosket and Short, 1973; Jouannean and de Marsee, 1973). RNA synthesis, however,appears to be under the control of cytokinin.

Raychoudhury *et al.*, (1965), using isolated nuclei from coconut milk, demonstrated that incorporation of labelled precursor into RNA was increased in the presence of kinetin. It was also shown in *Lemna minor* that [$^{32}PO_4^{2-}$] incorporation into nucleic acids was stimulated by benzyladenine treatment (Van Overbeek *et al.*, 1967). Trewavas (1970), however, found that benzyladenine stimulated both RNA synthesis and breakdown, thus increasing the rate of RNA turnover rather than the level of RNA. The effect of cytokinin on RNA synthesis in an *in vitro* system containing purified pea chromatin and *E.coli* RNA polymerase has been studied by Matthysee and Abrams (1970). They found that in the presence of a particular protein fraction, kinetin stimulated RNA synthesis.

In some cases, cytokinin not only influenced RNA synthesis but also protein synthesis. Fankhauser and Erismann (1969) found that both RNA and protein levels were increased as early as 15 minutes after addition of kinetin. Cytokinins have also been shown to influence the formation of a number of specific enzyme activities. The activity of tyramine methylpherase in roots of germinating barley embryos (Steinhart,

1964), the formation of α-amylase in *Phaseolus vulgaris* (Clum, 1967) and the rate of synthesis of carboxydismutase and NADP-dependent glyceraldehydediphosphate dehydrogenase in rye seedlings (Feierabend, 1969) appear to be under the influence of cytokinins.

The regulation of polyribosome formation by cytokinin treatment has been studied. Short *et al.*, (1974), using cultured cells of *Glycine max*, found that the polyribosome level doubled within 24 hours after treatment with zeatin compared to untreated controls. The peak of the cytokinin-induced stimulation of polysomes occurred within six hours after the treatment. Since initial stimulation of polysome formation by cytokinin was not blocked by actinomycin D, Short *et al.*, (1974) proposed that cytokinins stimulate polysome formation in the absence of additional RNA synthesis by modifying existing inactive monoribosomes so that they could complex with cytoplasmic mRNA particles and reinitiate protein synthesis.

Cytokinins are also able to modify ribosomes. Ralph *et al.*, (1972) reported that cytokinin inhibited a protein kinase which phosphorylated Chinese cabbage leaf ribosomal proteins. Although the role of ribosomal protein phosphorylation in polysome formation and protein synthesis is not known, there is a possibility that highly phosphorylated monoribosomes may represent storage ribosomes which are unable to initiate protein synthesis. By inhibiting ribosomal protein phosphorylation, cytokinins may prevent the formation of storage ribosomes and thus increase the percentage of ribosomes that are available for the formation of polyribosomes and eventually increase the protein synthetic activity.

The above biochemical studies on the mechanism of cytokinin action,

however, do not provide a clear pattern of reactions that could explain the cytokinin phenomenon. Much effort has been invested in searching for direct biochemical responses, such as the effects of cytokinin on DNA, RNA and proteins synthesis or on the activation, and the incorporation of cytokinins into RNA. This approach, while generating information, is not a total approach. An alternative line of study which could be more profitable is a "Metabolic Approach," that is, to follow the metabolic fate of the compound.

In an organism or even in a single cell, metabolic reactions are related to each other and any disturbance occurring to one metabolite will probably affect the rest of the cell's metabolism. This line of thought has recently been advanced by Hall. In his review, (Hall, 1973), he suggests that phenotypic expression should be considered as the consequence of the total interactions of all the molecular processes of the cell or tissue. Phenotypic expressions come not from the existence of the cytokinin molecule in the cell as a static entity but rather from its interactive processes with other molecules. When cytokinin enters the cell every molecular interaction is modified so that the total expression of the tissue is changed. Hall has also given the analogy of adding a pinch of a powerful dye to an already coloured paint, which results in a perceptible shift in hue. This model allows for what appears to be hormone-hormone interaction, as with coloured paint, one can obtain an almost infinite shading of hues by adding traces of two or three dyes in varying proportions. This concept emphasizes the interrelationship between molecules rather than the molecules themselves.

The present study was based on these considerations.

In order to understand the metabolic interrelationship among molecules the metabolism of cytokinin has to be established. Because not all the metabolic events of a natural-occurring cytokinin in any plant tissue are known, the immediate objective of this study was to establish the metabolic pathways of i^6Ado in the present experimental plant tissue, a tobacco callus, *Nicotiana tabacum,* Wisconsin No. 38.

As a prerequisite for understanding of the metabolism of a compound, the nature and the rate of its uptake by the experimental tissue, in conjunction with the rate of utilization of the compound inside the cell, needs to be studied. The biological activity of the cytokinin not only depends on the chemical structure but also on the availability of the compound. Since we are dealing with an exogenous source of the compound, the transport mechanism across the membrane or simply the uptake process of the compound by the experimental tissue ought to be taken into consideration. Available evidence indicates that there may be some correlation between the rate of uptake and the metabolic transformation of a compound inside the cell. However, at present, neither the rate of i^6Ado uptake nor its rate of metabolism in any plant system is known. Therefore, the second objective of this study was to investigate a possible relationship between the rate of i^6Ado uptake and its metabolism in the tobacco callus.

Finally, the nature of the interrelationship between cytokinin and auxin and between cytokinin and gibberellic acid was explored. Although different ratios of these compounds have been shown to give different physiological responses, the exact nature of biochemical interaction involved has never been studied in depth. Using radioactive i^6Ado as a

precursor and different concentrations of auxin and ABA, the influence of the latter two hormones on the metabolism of i^6Ado was studied.

II. MATERIALS AND METHODS

MATERIALS

(1) Biologicals:

In this study, two types of tobacco pith, the normal cytokinin - auxin dependent, and the autonomous, cytokinin - auxin independent, were used. The normal plant hormone dependent tissue, designated as KX, originated from the stem of tobacco, *Nicotiana tabacum* var. Wisconsin No. 38 and the autonomous tissue was derived from the KX tissue. During the process of tissue culturing, a strain of callus was isolated and it was found to grow on a medium without any plant growth factors. It was named O-1.

(2) Chemicals:

Except for those listed below, all common chemicals were purchased either from Fisher Scientific Company, Fair Lawn, New Jersey, or J.T. Baker Chemical Co., Phillipsburg, New Jersey and were of certified A.C.S. or Baker analyzed reagents grades, respectively.

i-Inositol	:	General Biochemicals. Ohio
Thiamine HCl	:	" " "
Pyridoxine HCl	:	" " "
Zeatin	:	Calbiochem., California
Zeatin Riboside	:	" "
Kinetin Riboside	:	Sigma, St. Louis, Mo.
Napthaleneacetic acid	:	Eastman Organic Chemicals, N.Y.

N^6-(Δ^2-Isopentenyl-adenosine	:	Starks Associates Inc., Buffalo, N.Y.
Abscisic acid	:	A gift sample by Dr. A. Oaks, Department of Biology, McMaster University from Shell Research Limited, Sittingbourne, Kent, through Dr. B.V. Milborrow.

METHODS

(1) Synthesis and Identification of [8-^{14}C]N^6-(Δ^2-Isopentenyl)adenosine

[8-^{14}C]Adenosine (specific activity 47 mCi/mmol) was purchased from Amersham/Searle Co.. The synthesis of [8-^{14}C]i^6Ado was performed according to the method described by Paces *et al.* (1971) modified as follows: 50 μCi of [8-^{14}C]Ado was dried in a desiccator and mixed with unlabelled Ado in 10 μl of dimethylformamide (DMF) to make a final concentration of 0.2M. Ten μl of freshly distilled Δ^2-isopentenyl bromide (Columbia Organic Chemicals) in DMF (2.2M solution) was added to the above reaction vessel, which also contained 3 mg of oven dried $CaCO_3$. The reaction was allowed to proceed at room temperature for 40 hours in darkness, with occasional shaking. The $CaCO_3$ was separated from the reaction mixture and was thoroughly washed with DMF (5 x 100 μl). The combined solutions were evaporated in another reaction vial. Fifth μl each of methanol and 25% dimethylamine was added to the vial and the reaction was allowed to proceed for another 40 hours at room temperature in darkness. At the end of the second reaction, the solution was evaporated under reduced pressure and the residue was redissolved in 35% ethanol.

The reaction products were separated by means of chromatography on a Sephadex LH-20 column, using 35% ethanol as the eluting solvent. The fractions containing [8-^{14}C] i^{6}Ado were pooled, evaporated and redissolved in 35% ethanol. The product was rechromatographed on a second LH-20 column in 35% ethanol. The purity of the [8-^{14}C] i^{6}Ado was checked by co-chromatography with an authentic sample of unlabelled i^{6}Ado by TLC ($CHCl_3$: CH_3OH = 9 : 1) and by paper chromatography, using solvent systems A and E (vide infra, p. 23)

In order to confirm the validity of the experimental procedure a sample of unlabelled i^{6}Ado was prepared on the same scale, and the identity of the final product was determined by gas-liquid chromatography and mass spectroscopy. This product was identical to authentic i^{6}Ado in both determinations. The mass spectrum of the synthetic sample is shown in Figure 1.

(2) Growth and Maintenance of Tobacco Callus Tissue

The normal hormone dependent tobacco pith tissue (*Nicotiana tabacum* L. var. Wisconsin No. 38) and the derived autonomous tissue were grown on the following medium as originally described by Murashige and Skoog (1962).

Culture Solutions for KX and 0 - 1 tissues

Each basic solution has a volume of 1 ℓ.

Solution (A) : 50 mg NH_4NO_3

Solution (B) : 50 g KNO_3

25 g $Ca(NO_3).4H_2O$

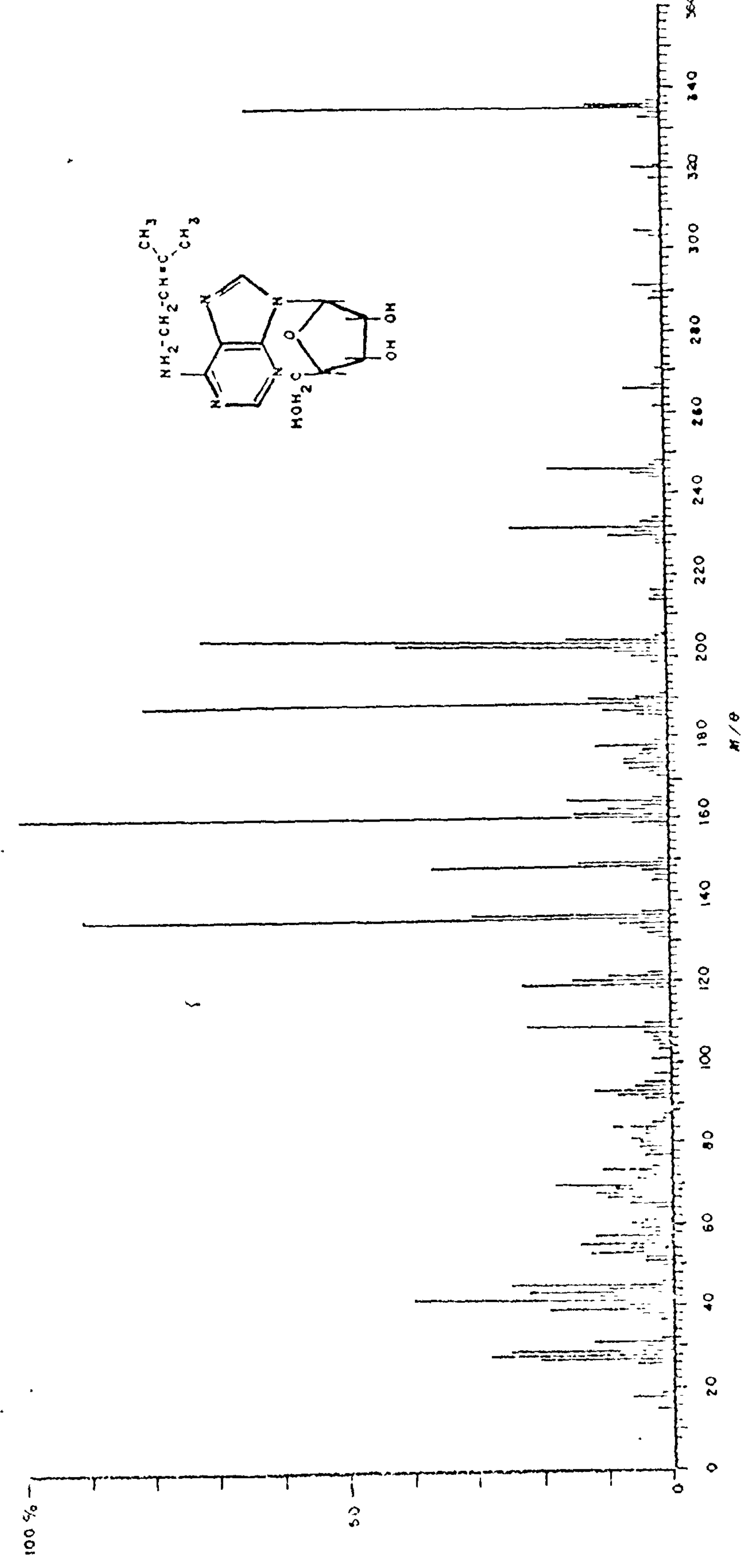
Solution (C) : 50 g KH_2PO_4

1.0 g H_3BO_3

0.16 g KI

Figure 1. Mass spectrum of the synthetic N^6-(Δ^2-isopentenyl)adenosine

Spectrum obtained at 70 ev, direct inlet, 185 C. The molecular ion occurs at m/e 335; loss of CH_3 at 320; loss of $C(CH_3)_2$ and H at 292. The free base occurs at 203; loss of CH_3 at 188; loss of adenine at 135. The most abundant species, 160 is due to loss of ribosyl and $C(CH_3)_2$ from i^6Ado.


M/e
100 %
50
0
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360

Solution (D) : 60.0 g $MgSO_4.7H_2O$

10.0 g KCl

1.5 g $ZnSO_4.7H_2O$

1.0 g $MnSO_4.H_2O$

Solution (E) : 0.02 g Thiamine HCl

0.1 g Nicotinic acid

0.1 g Pyridoxine HCl

0.4 g Glycine

Solution (F) : 6.0 g EDTA sodium salt

4.344 g $FeCl_3$

<u>Basal Medium for KX tissue</u>

(i)	Solution (A)	=	20.0 ml
(ii)	Solution (B)	=	20.0 ml
(iii)	Solution (C)	=	5.0 ml
(iv)	Solution (D)	=	5.0 ml
(v)	Solution (E)	=	5.0 ml
(vi)	Solution (F)	=	5.0 ml
(vii)	i-Inositol	=	100.0 mg
(viii)	Sucrose	=	30.0 g
(ix)	Agar	=	10.0 g

The volume was made up to about 800 ml with distilled water and the solution was adjusted to pH 5.8 with 2.0 N NaOH. Twenty ml of NAA solution (50.0 mg/ℓ) and 10.0 ml ribosyl-kinetin solution (108 mg/ℓ) were added and the volume was made up to 1 ℓ with distilled water. The mixture was heated, with constant stirring, until a clear solution was obtained. About 100 ml of this solution was transferred into culture bottles,

which were then sterilized.

Basal Medium for 0 - 1 tissue

The basal medium for the 0 - 1 tissue was the same as that for the KX tissue, except that the two plant hormones, NAA and ribosyl kinetin, were omitted.

Tissues were generally transferred to new medium every three to four weeks. They were maintained in a growth chamber, in total darkness at 27°C. Eight to 16 day old tissues, believed to be in an expotential growth period, were used in this study. Only friable tissue was selected for each experiment.

(3) Incubation of Tissues

Two different conditions were used for incubation of tissues. Buffer A. For the metabolic transformation of i^6Ado or the qualitative studies, the tissue was incubated in 0.05M phosphate buffer, pH 7.0, containing 0.005M $MgCl_2$ at 37°C. Buffer B. In later comparative studies between KX and 0 - 1 tissues or the quantitative studies, the tissue was incubated in its own basal medium without agar, under sterilized conditions at 27°C. There were no differences between the two conditions as far as the type and the number of compounds, however, a greater yield of products was obtained using buffer A.

A. Qualitative Studies

Five g of tissue were preincubated in 10.0 ml of buffer A for one hour at 37°C. The suspension was gently agitated during incubation. A measured amount of [8-^{14}C] i^6Ado was added to the medium and incubation was continued for another five hours. At the end of the incubation period, the mixture was filtered through a Millipore filter (SSWP 04700).

The tissue was thoroughly washed five times with 10.0 ml of 0.5M phosphate buffer, pH 7.0, containing 50 μM unlabelled i^6Ado.

B. Quantitative Studies

The same procedure used in the qualitative studies was applied, except for the following modifications. The incubation medium was changed to buffer B and the preincubation period was extended to four hours. The incubation temperature was reduced from 37°C to 27°C (the same temperature as the growth chamber). All manipulations were done under sterile conditions.

After incubation, the tissue was filtered and washed with buffer B and the combined filtrate and washings collected.

(4) Determination of Metabolites

Since the starting compound, [8-^{14}C]i^6Ado was labelled, all metabolites containing the C-8 group were radioactive. The radioactive profile from LH-20 columns and all other individual measurements were determined by a Nuclear Chicago Mark I liquid scintillation counter. Xylene : Triton X-114 (75 : 25) solvent system with 3 g PPO/l and 0.2 gm POPOP/l was used as scintillation fluid. Radioactive spots on paper chromatograms and on electrophoresis paper were located by scanning in a Nuclear Chicago Actigraph III instrument.

After locating a compound on paper, it was eluted by a centrifugation technique. The located compound was cut out and the paper was soaked in distilled water. It was then centrifuged in a polyethylene thimble, contained in a 15 ml conical centrifuged tube, using a clinical centrifuge running for three minutes at maximum speed. The eluted solution was collected and the soaking and centrifugation procedure was

repeated twice. All eluents were combined, evaporated under reduced pressure and the residue was redissolved in appropriate solvent for further determination. This procedure elutes more than 95% of radioactive materials from paper.

(5) Extraction of Metabolites

The washed tissue containing unlabelled i^6Ado was immediately transferred into 10 ml of 80% ethanol in an all glass tissue grinder tube. The tissue was ground for five minutes using a Potter-Elvehjem homogenizer. The suspension was centrifuged for 10 minutes at 10,000 x g. The supernatant was collected and the residue was re-extracted two more times with 5 ml of 80% ethanol. The combined supernatants are termed the ethanol soluble metabolites.

The residue remaining after the third extraction is termed the ethanol insoluble fraction. To determine the radioactivity in it, the residue was suspended in 2 ml of 2.0 N KOH contained in a 15 ml centrifuge tube. The suspension was agitated for 24 hours at 50°C. Three ml of 2.0 N HCl was added and hydrolysis was continued for another 24 hours. After the second hydrolysis, the suspension was centrifuged in a clinical centrifuge at maximum speed for 5 minutes. The supernatant was used for determination of the amount of radioactivity left in the ethanol insoluble fraction.

The thoroughness of the extraction procedure in removing ethanol soluble radioactive metabolites was tested for five successive extractions. It was found that after three extractions more than 95% of the total extractable ethanol soluble materials were removed. For this reason the tissue was extracted only three times.

(6) Separation of Metabolites

The primary separation of i^6Ado and its metabolites was by Sephadex LH-20 (Pharmacia Co.) column chromatography (Armstrong *et al.* 1969). Secondary separation was by paper and thin layer chromatography and by high voltage paper electrophoresis. A longer LH-20 column (2.5 x 80.0 cm) was used in order to obtain better separation. Whatman No. 3 MM chromatographic papers were first washed with 0.02 N HCl and then with distilled water, before they were used.

The solvent systems were:

A 2-PrOH : NH_3 : H_2O = 7 : 1 : 2

B 1-BuOH : HOAc : H_2O = 12 : 3 : 5

C 1-BuOH : HOFc : H_2O = 10 : 4 : 5 (Upper Phase)

D 1-PrOH : EtOAc : H_2O = 1 : 4 : 5 (Upper Phase)

E 2-PrOH : conc.HCl : H_2O = 680 : 170 : 144

Typical R_f values are given in Table 1 (see below).

The TLC plates were developed in CH_3OH : $CHCl_3$ (1 : 9) (Playtis *et al.* 1971) and paper electrophoresis was performed in Tris/citric acid buffer (0.05M, pH 3.5) according to the method described by Doree and Terrine (1973).

(7) Identification of Metabolites

The identification of i^6Ado and its metabolites was based on the following criteria.

(a) The elution profile of the Sephadex LH-20 column was divided into five fractions according to the position of the reference compounds on the standard profile. Figure 2 shows a typical elution profile of a sample and some of the reference compounds.

Figure 2. The elution profile of i^6Ado and its related compounds.

Five g of KX tissue were preincubated in 10 ml of phosphate buffer (0.05 M, pH 7.0) containing 0.005 M $MgCl_2$ for 1 hour at 37°C. About 5.75×10^5 cpm at [8-^{14}C] i^6Ado (Sp. Act. 23.5 mCi/mmol) was added and the incubated was continued for 5 hours (final concentration of i^6Ado in the medium was 1.3×10^{-6}M). The 80% ethanol soluble [^{14}C]-metabolites were fractionated on a LH-20 column (2.5 x 80 cm), using 35% ethanol as eluant. Fractions of 10 ml were collected and 0.5 ml from each fraction was taken for scintillation counting. The radioactive peaks were designated as fraction I to V. Duplicate samples were run for each experiment, which were repeated more than three times with a similar pattern of results being obtained.

The lower diagram represents the position of reference compounds, run on the same LH-20 column under the same conditions.

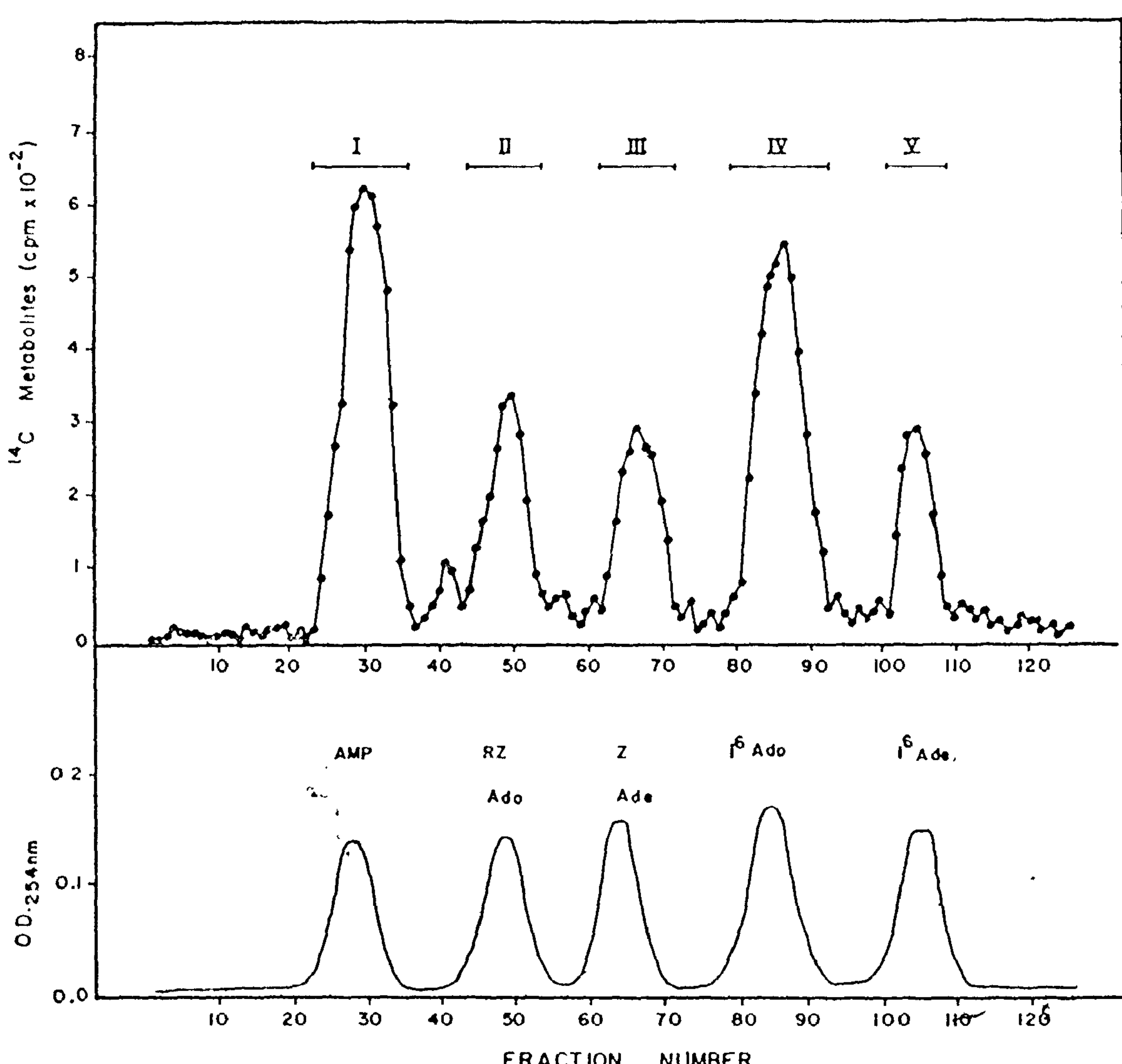


Figure 2

(b) These five fractions were separated into individual components by means of paper chromatography using two to three solvent systems. Typical R_f values of the standard compounds determined under the same conditions as the unknowns are shown in Table 1.

(c) The following chemical and enzymatic treatments were applied to labelled products in order to study their reactions. These chemical and enzymatic transformations have already been studied with reference compounds under identical conditions. In other words, their derivatives were used to confirm the starting compounds by known reactions. For the identification of these compounds, not only paper chromatography but TLC and high voltage paper electrophoresis were also applied.

(i) Treatment with 0.1% $KMnO_4$

This reaction removes the Δ^2-isopentenyl side chain from i^6Ado. The reaction conditions are so mild that they have been used to remove the side chain from the modified nucleoside residue of tRNA without damaging the rest of the molecule (Hall, 1971). In this study the reaction has been exploited, not only for i^6Ado, but also for its base, nucleotide and zeatin derivatives.

For a typical reaction 0.25 ml of 0.1% $KMnO_4$ was added to 0.5 ml of the sample in water and the reaction was allowed to proceed for five minutes. One ml of 95% ethanol was added to stop the reaction and the solution was left for another 24 hours at room temperature. The supernatant was collected by centrifugation and was evaporated under reduced pressure. The residue was redissolved in 0.5 ml of 35% ethanol and the products were resolved by means of paper chromatography.

TABLE 1

Typical R_f values of reference compounds

(With Whatman No. 3 MM Paper)

Compounds	Solvent Systems				
	A	B	C	D	E
Ade	0.46	0.59	0.23	0.24	0.26
Ado	0.51	0.48	0.12	0.17	0.31
Inosine	0.37	0.44	-	-	0.18
Z	0.70	0.76	0.49	0.52	0.60
RZ	0.78	0.74	0.40	0.55	0.67
i^6Ade	0.88	0.89	0.86	0.80	0.77
i^6Ado	0.89	0.86	0.83	0.81	0.84
AMP	0.08	0.10	0.10	-	-

Solvent Systems A : 2-PrOH : NH_4OH : H_2O = 7 : 1 : 2

B : 1-BuOH : HOAc : H_2O = 12 : 3 : 5

C : 1-BuOH : HOFc : H_2O = 10 : 4 : 5 (Upper phase)

D : 1-BuOH : EtOAC : H_2O = 1 : 4 : 2 (Upper phase)

E : 2-PrOH : Conc. HCl : H_2O = 680 : 170 : 144

(ii) Treatment with Alkaline Phosphatase

Calf intestinal alkaline phosphatase was purchased from Calbiochem. Ten units of the enzyme solution (0.5 ml) was added to the sample in 0.5 ml of Tris-HCl buffer (0.05M, pH 8.6) containing 0.005M $MgCl_2$. The solution was incubated for two hours at 37°C. Three ml of 95% ethanol was added and the solution was centrifuged. The supernatant was evaporated under reduced pressure and the residue was dissolved, chromatographed on paper chromatography and subjected to electrophoresis.

(iii) Treatment with Nucleotidases

5'-Nucleotidase and 3'-nucleotidase were obtained from Sigma. About three units of the former and about two units of the latter were used. The experimental procedure was essentially the same as in the treatment with alkaline phosphatase.

(iv) Treatment with Adenosine Aminohydrolase

Adenosine aminohydrolase or adenosine deaminase was from Calbiochem. The deamination reaction was performed according to the method described by Hall *et al.*, (1971). About two units of the enzyme solution (0.5 ml) was added to a sample in 0.5 ml of Tris/HCl buffer (0.05M, pH 7.5) containing 0.005M $MgCl_2$ and 0.01M 2-mercaptoethanol. The incubation was carried out for five hours at 37°C and the reaction was stopped by adding 3 ml of 95% ethanol. The supernatant collected after centrifugation was evaporated under reduced pressure. The residue was redissolved in appropriate solution for further determinations.

(v) Treatment with Partially Purified Cytokinin Oxidase from Corn Extract.

The presence of this enzyme was first described by Paces *et al.*,

(1971) in a crude extract of tobacco tissue (*Nicotiana tabacum* var. Wisconsin No. 38) and was later found in corn kernels by Whitty and Hall (1974).

To prepare an enzyme extract, frozen corn kernels were ground in a grinder, which was chilled with liquid nitrogen. Four hundred g of corn powder were thoroughly stirred with 800 ml of phosphate buffer (0.05M, pH 6.8) containing 0.005M $MgCl_2$ and 0.005M 2-mercaptoethanol for one hour at room temperature. The mixture was centrifuged at 10,000 x g for 30 minutes and chilled acetone was added to the supernatant at 4°C. The precipitate that formed between 40 and 60 percent acetone saturation was collected and dissolved in 50 ml of Tris/HCl buffer (0.05M, pH 7.5) containing 0.005M $MgCl_2$ and 0.005M 2-mercapto-ethanol. The solution was dialyzed against one ℓ of the same buffer for 24 hours at 4°C and was centrifuged for 30 minutes at 10,000 x g. The supernatant fraction was used as the enzyme extract for the cleavage activity of N^6-substituted adenine or adenosine.

A sample was dissolved in 0.5 ml of Tris/HCl buffer (0.05M, pH 7.5)containing 0.005M $MgCl_2$ and 0.5 ml of partially purified corn enzyme extract (about 4.6 O.D./ml) was added. It was incubated for five hours at 37°C. About 3 ml of 95% ethanol was added and the precipitated protein was removed by centrifugation. The supernatant was evaporated under reduced pressure and dissolved in the appropriate solvent for chromatographic determinations.

III. RESULTS

The results of this study are presented under three headings.

(1) Qualitative studies

(2) Quantitative studies

(3) Interhormonal effect studies

(1) Qualitative Studies

The study of the physiological actions of i^6Ado in tobacco pith is well documented. Generally, every physiological response is the result of biochemical actions occurring inside the cells so the metabolism of i^6Ado is of interest. The main purpose of the studies in this section was to examine both KX and O-1 tissues for the presence of i^6Ado and its derivatives, that is by inference the presence of enzyme systems that can metabolize i^6Ado into different compounds. From the metabolites present the metabolic pathways of i^6Ado in these tissues can be suggested.

In order to establish the metabolic pathways, the metabolites have to be identified. To be able to identify these compounds they have to be separated first into individual components and then the individual compounds must be identified by appropriate methods.

(i) Column chromatographic separation of ethanol soluble metabolites

In all qualitative studies only the ethanol soluble fraction was analyzed. Figure 2 shows a typical elution profile of ethanol extractable metabolites from KX tissue on the LH-20 column. It can be seen that five

radioactive peaks predominate, named fraction I to V. A similar profile was obtained with 0 - 1 tissue. The peaks were tentatively classified as nucleotide fraction, nucleoside fraction, base fraction, modified nucleoside fraction and modified base fraction, respectively, based on the position of some of the reference compounds on the elution profile by the same column (Figure 2, lower diagram).

Since these fractions could contain more than one compound, fractions I to V were further separated into individual components by paper chromatography in an attempt to identify all the possible metabolites.

(ii) Paper chromatographic separation of fraction I

Two samples of fraction I were analyzed by paper chromatography using solvent systems A and B. In order to detect radioactive materials, the developed papers were scanned in a Nuclear Chicago Actigraph III. In both determinations, four radioactive peaks were determined, designated peak Nos. 1, 2, 3 and 4 (Figure 3). A sample of authentic AMP was also run on the same paper in both systems. It was found that the R_f value of peak No. 1 coincided with that of AMP in both determinations, which suggests that it was AMP. Confirmatory studies were not carried out.

Peak No. 4 in Figure 3A was suspected of being i^6AMP, because the R_f value of this nucleotide was found (by Hacker, 1970) to be 0.45 in this solvent system. It was eluted with distilled water by a centrifugation technique and rechromatographed on paper in solvent system B. It migrated as a single peak, which coincided with the position of peak No. 4 in Figure 3B. Confirmatory studies of this compound were carried out as described in a later section.

Figure 3. Paper chromatographic separation of fraction I

Two samples, about 30,000 cpm each, of fraction I from Figure 1 were streaked on papers, which were then chromatographed in solvent systems A and B for 16 to 18 hours in the descending manner. The papers were scanned in a Nuclear Chicago Actigraph III to detect radioactive compounds. The efficiency of counting was about 9%. Authentic samples of AMP were also run on both papers and were found to coincide with radioactive peak No. 1 in both systems. A : Solvent system A; B : solvent system B.

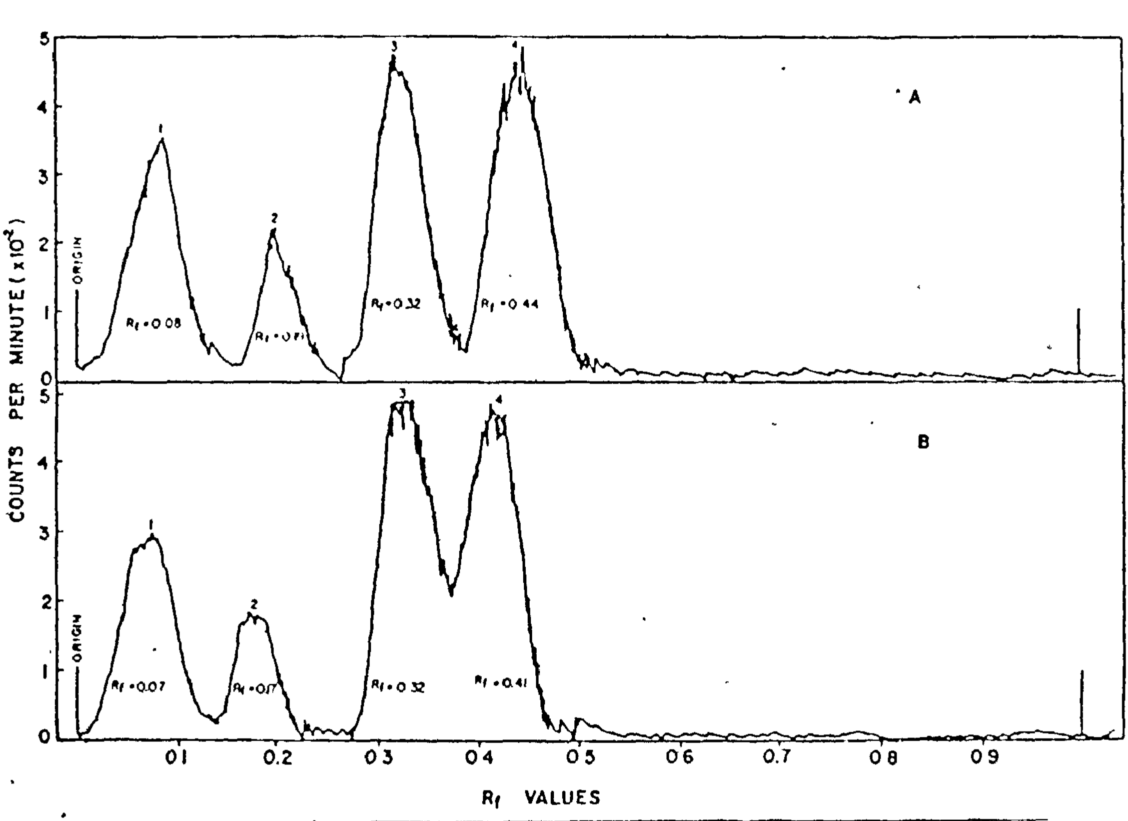


Figure 3

Peak No. 3 in Figure 3B was also suspected of being the nucleotide of RZ, because its R_f value was reported as 0.32 in this solvent system (Sondheimer and Tzou, 1971). The sample was eluted and rechromatographed on paper in solvent system A. A single peak, which migrated at the same position as peak No. 3 in Figure 3A, was found. Confirmation of the identity of this compound was carried out and the data are shown in a later section.

Peak No. 2, however, was not identified.

(iii) Confirmation studies of peak No. 3 (Figure 3A) as i^6AMP

(a) $KMnO_4$ oxidation: A sample of material from peak No. 4 (Figure 3), was treated with 0.1% $KMnO_4$. The products were chromatographed on paper in solvent system A and the results, after scanning in an Actigraph III, are shown in Figure 4B. There were three radioactive peaks, one of which was the starting compound (R_f = 0.49). The majority of the radioactivity was found at an R_f of 0.09, which coincided with the position of AMP. A third product (R_f = 0.35) was also obtained. Figure 4A shows a control sample of peak No. 4 (Figure 4), which had not undergone $KMnO_4$ oxidation.

The removal of Δ^2-isopentenyl side chain from the modified nucleoside residue of tRNA (Hall, 1971) was accomplished by the following reaction:

Figure 4. $KMnO_4$ oxidation of suspected i^6AMP

The suspected sample of i^6AMP (about 8,000 cpm of material from peak No. 4 from Figure 3) was dissolved in 0.5 ml of distilled water and 0.25 ml of an aqueous solution in 0.1% $KMnO_4$ was added. After 15 minutes at room temperature 1.0 ml of 95% ethanol was added and the solution was left for 24 hours at room temperature. The reaction products were chromatographed on paper in solvent system A. A: Untreated sample. B: The sample after $KMnO_4$ oxidation.

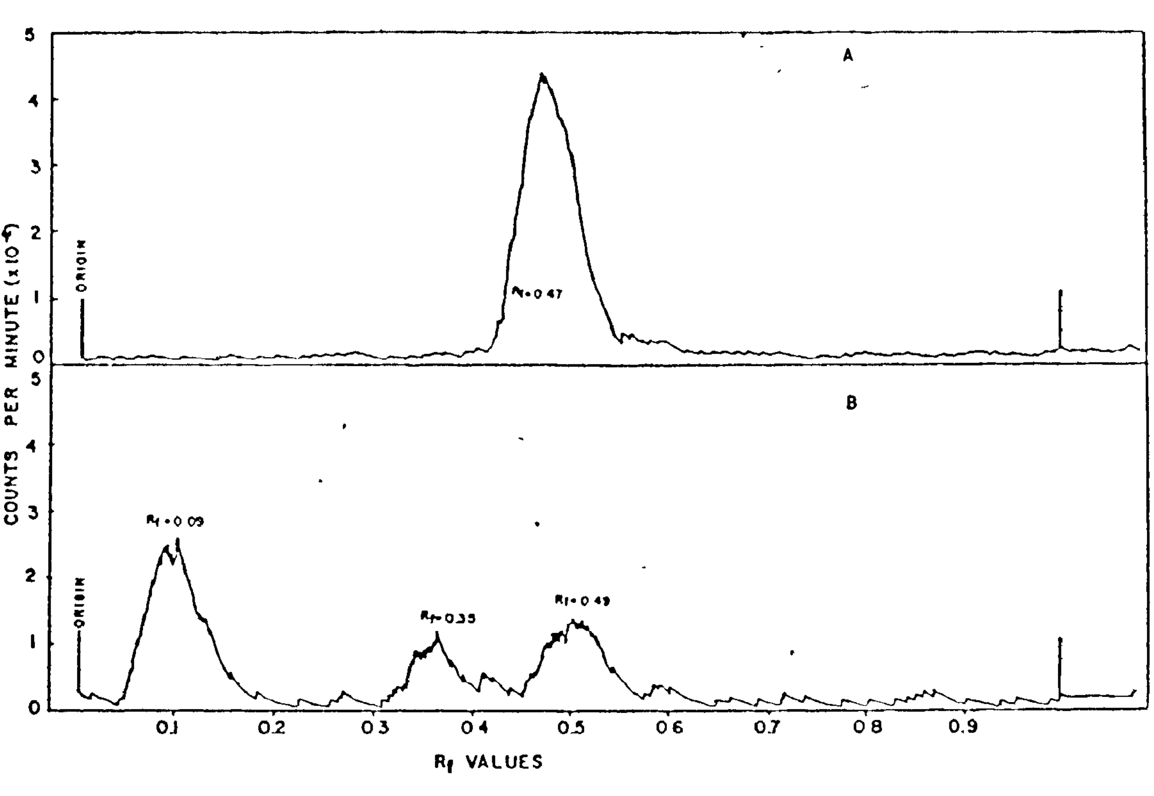


Figure 4

$i^6AMP \xrightarrow{0.1\%\ KMnO_4} AMP$

It can be seen that after the $KMnO_4$ oxidation (Figure 4B), the majority of the radioactive materials shifted to a new position, which coincides with that of AMP.

(b) Paper electrophoresis: Another sample of peak No. 4 (Figure 3) was also treated with 0.1% $KMnO_4$. The products were then separated by paper electrophoresis in a Tris/citric acid buffer. An untreated sample, and an authentic AMP were run on the same paper as the controls. The results are shown in Figure 5.

The sample after $KMnO_4$ oxidation migrated at 2.98 cm/hr, a rate identical to that of authentic AMP. The untreated control sample gave a mobility of 3.38 cm/hr.

(c) Treatment with phosphatase enzymes: Three samples of peak No. 4 (Figure 3) were treated with intestinal alkaline phosphatase, 5'-nucleotidase and 3'-nucleotidase, respectively. The products were examined by paper chromatography in solvent system A. An untreated sample and a sample of authentic i^6Ado were included on the same paper as controls.

Both alkaline phosphatase and 5'-nucleotidase cleaved off the phosphate group and a product (R_f = 0.90), which coincided with

Figure 5. Paper electrophoresis of putative i^6AMP

The sample of presumed i^6AMP (about 4,500 cpm of peak No. 4 from Figure 4) was treated with 0.1% $KMnO_4$ as described in Figure 4. The products were separated by paper electrophoresis in a Tris/Citric acid buffer (0.05 M, pH 3.5) at 60 V/cm for 5 hours. An untreated sample of peak No. 4 was run on the same paper. A : The result of untreated control; B : the same compound treated with $KMnO_4$.

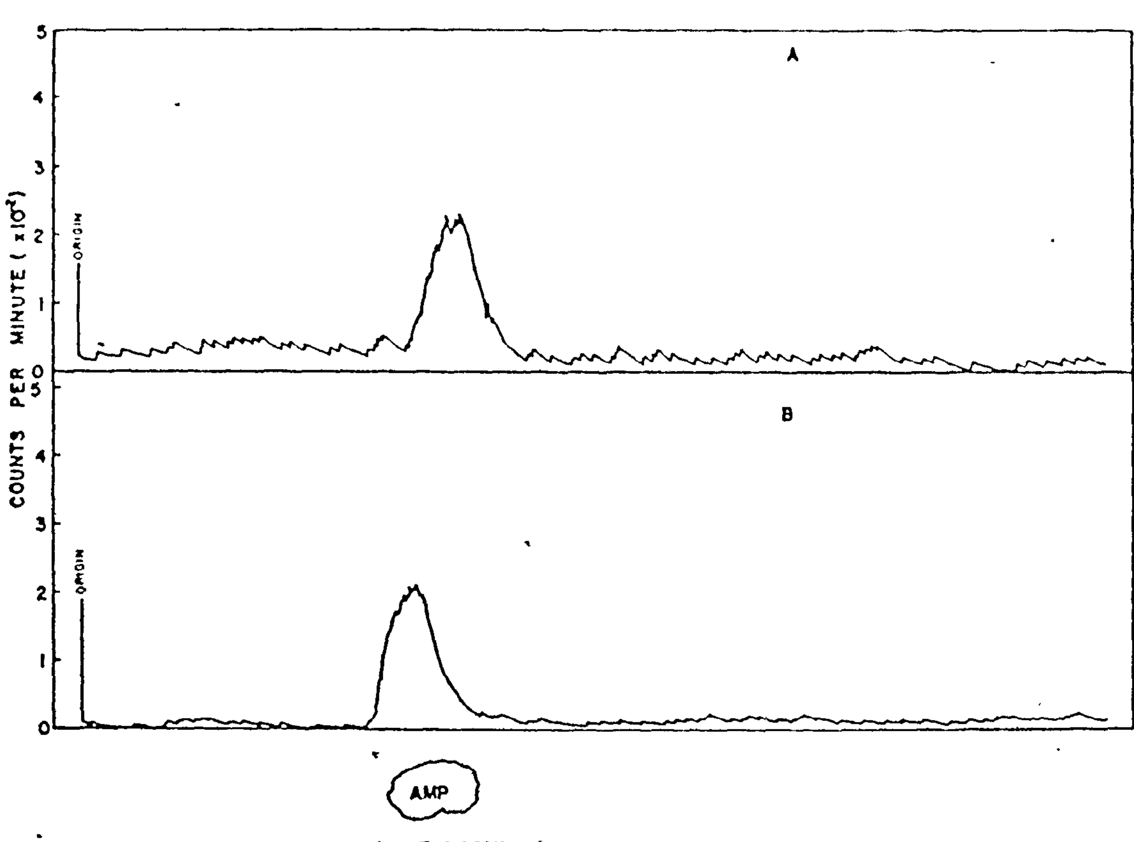


Figure 5

authentic i^6Ado, was obtained (Figure 6, B and D). However, the control and the 3'-nucleotidase treated sample were unchanged (R_f = 0.46). The identity of the presumed i^6Ado from the enzyme treated samples of the nucleotide were further confirmed by paper chromatography in different solvent systems, and results similar to the diagrams as shown in Figures 16A, B and C were obtained.

From these experiments it was concluded that the material from peak No. 4 (Figure 3) contained the Δ^2-isopentenyl side chain at the N-6 position of the modified nucleotide and a phosphate group, located at the 5'- position of the ribose group. The compound was i^6Ado - 5' - P or simply i^6AMP.

(iv) Confirmation studies of peak No. 3 (Figure 3B) as a nucleotide of RZ

Experiments similar to those used to confirm the identity of i^6AMP, were applied to the sample of radioactive peak No. 3 (Figure 3B). The results are shown in Figures 7, 8 and 9. The identity of RZ obtained from the enzyme treated sample of the nucleotide was further confirmed by paper chromatography in different solvent systems and results similar to the diagrams as shown in Figures 10A, B and C were obtained. In addition, the RZ sample was confirmed as the *trans* isomer by means of TLC as shown in Table 2.

On the basis of these experiments it was concluded that peak No. 3 from Figure 3 is the 5'-nucleotide of *trans*-RZ.

(v) Paper chromatographic separation of fraction II

Three samples of fraction II from Figure 2 were further separated into individual components by paper chromatography in solvent systems A, B and C respectively. An authentic sample of RZ was included

Figure 6. Enzymatic treatment of a suspected sample of i^6AMP

Four 0.5 ml samples of the suspected i^6AMP (Figure 3) were dissolved in Tris/HCl buffer (0.05 M, pH 8.6) and different phosphatases were then added. The incubation was performed for 2 hours at 37°C and 95% ethanol was added to stop the reaction. The products were chromatographed on paper in solvent system A.

A : With no enzyme

B : With intestinal alkaline phosphatase

C : With 3'-nucleotidase

D : With 5'-nucleotidase

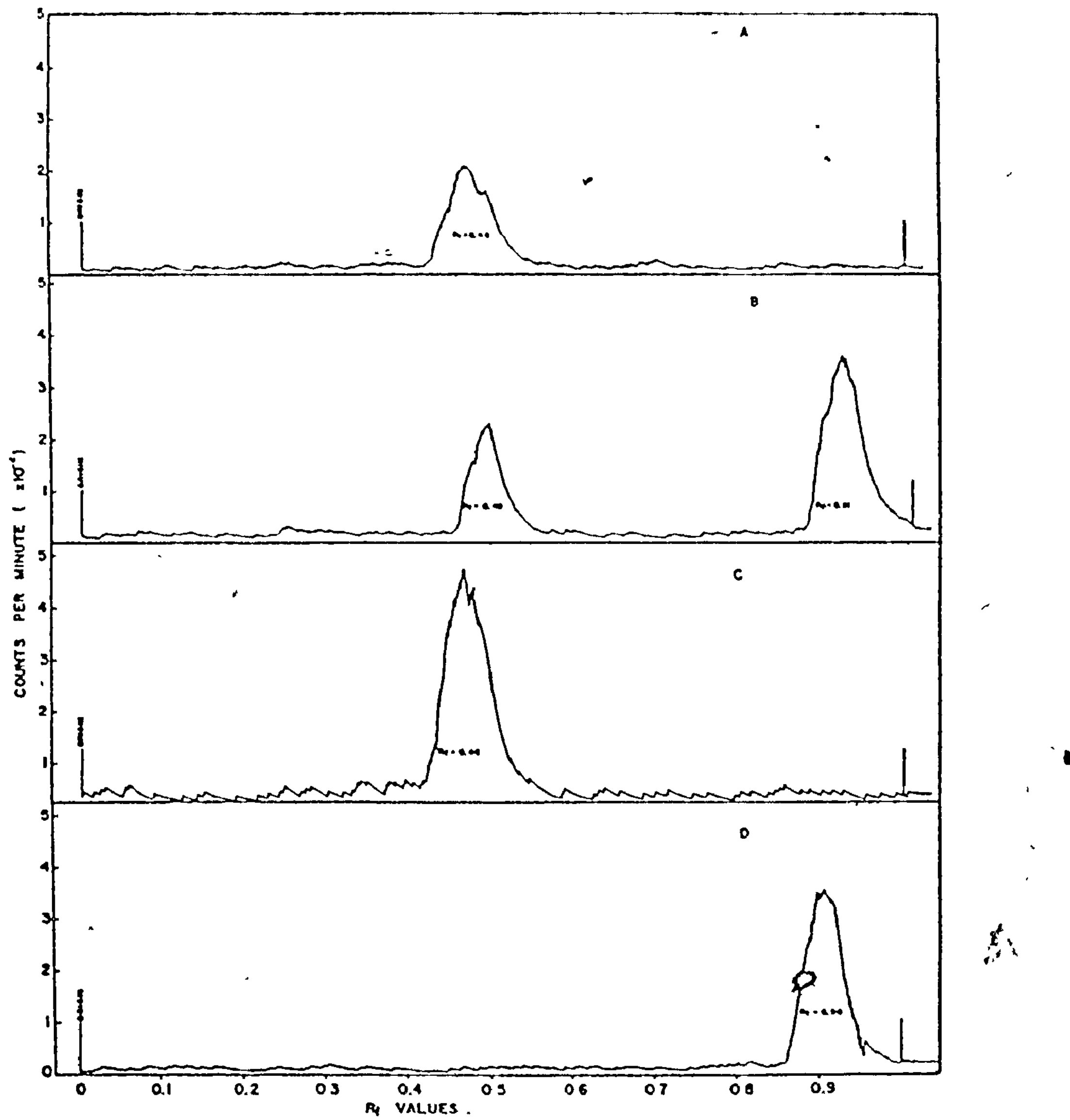


Figure 6

Figure 7. $KMnO_4$ oxidation of a suspected sample of RZ-nucleotide

The suspected sample of RZ-nucleotide (radioactive peak No. 3 from Figure 3) was dissolved in 0.5 ml of distilled water and the $KMnO_4$ oxidation was performed as described in Figure 4. The products were chromatographed on paper in solvent system B. A : Untreated control sample; B : the result after $KMnO_4$ oxidation.

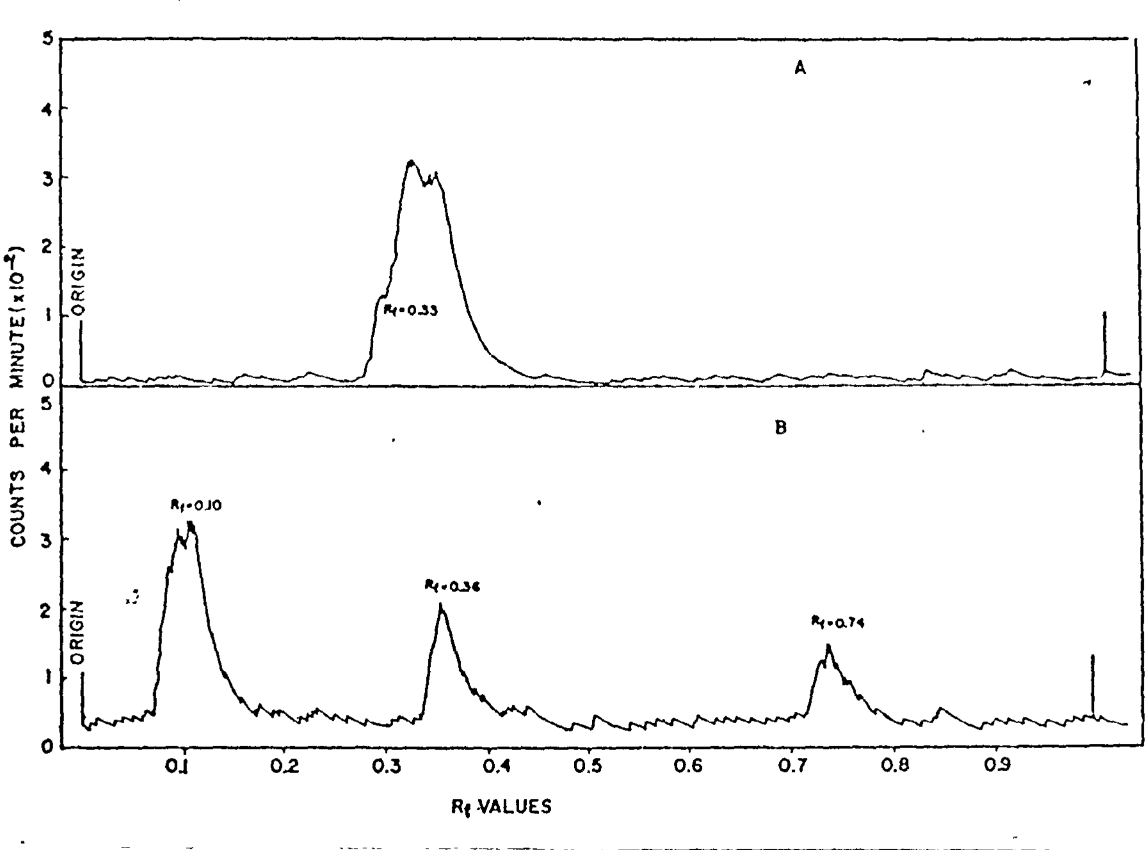


Figure 7

Figure 8. Paper electrophoresis of a suspected sample of RZ-nucleotide

A sample of suspected RZ-nucleotide (radioactive peak No. 3 from Figure 3) was treated with 0.1% $KMnO_4$ as described before. The products were separated by paper electrophoresis (60 V/cm) in Tris-Citric acid buffer (0.05 M, pH 3.5) for 5 hours. An untreated sample was run on the same paper. A : Untreated control sample; B : the sample after $KMnO_4$ oxidation.

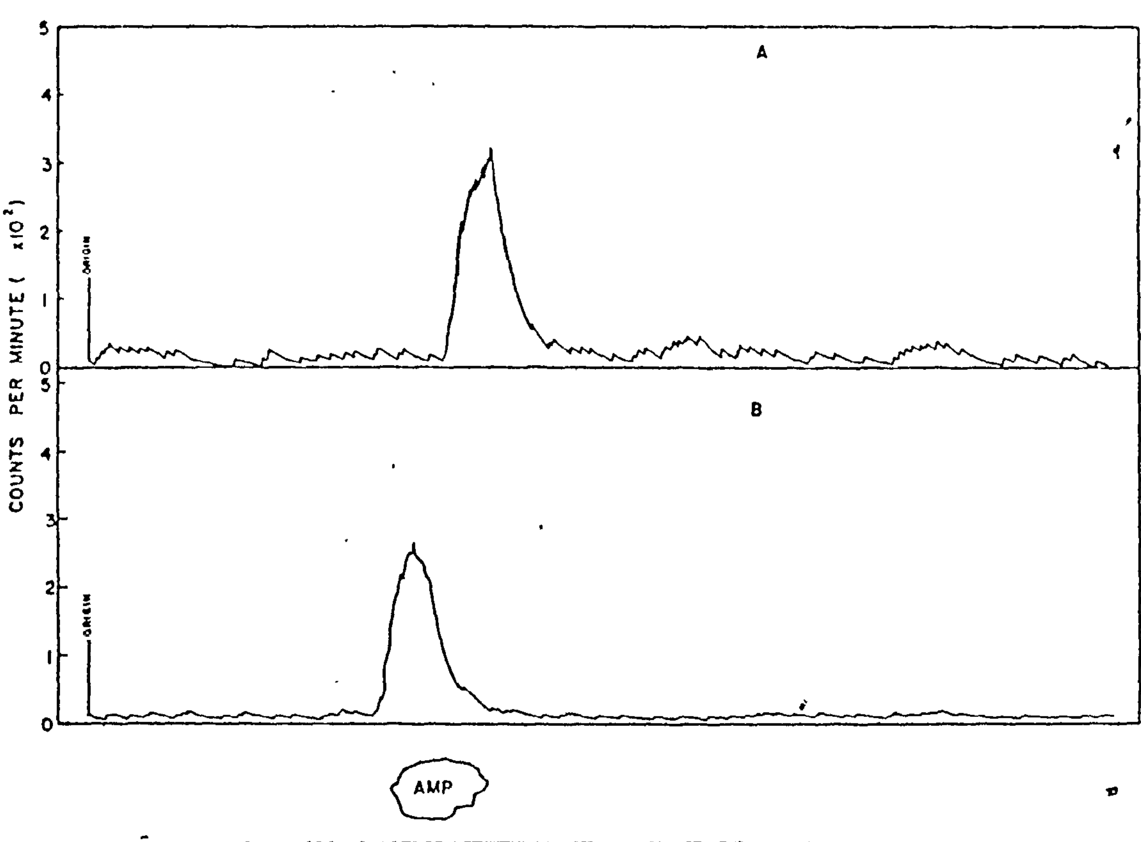


Figure 8

Figure 9. Enzymatic treatment of suspected samples of RZ-nucleotide

Four 0.5 ml samples of suspected RZ-nucleotide were each dissolved in Tris/HCl buffer (0.05 M, pH 8.6) and different phosphatases added. The samples were incubated for 2 hours at 37°C, after which 95% ethanol was added to stop the reaction. The products were chromatographed on paper in solvent system B.

A : With no enzyme

B : With intestinal alkaline phosphatase

C : With 3'-nucleotidase

D : With 5'-nucleotidase

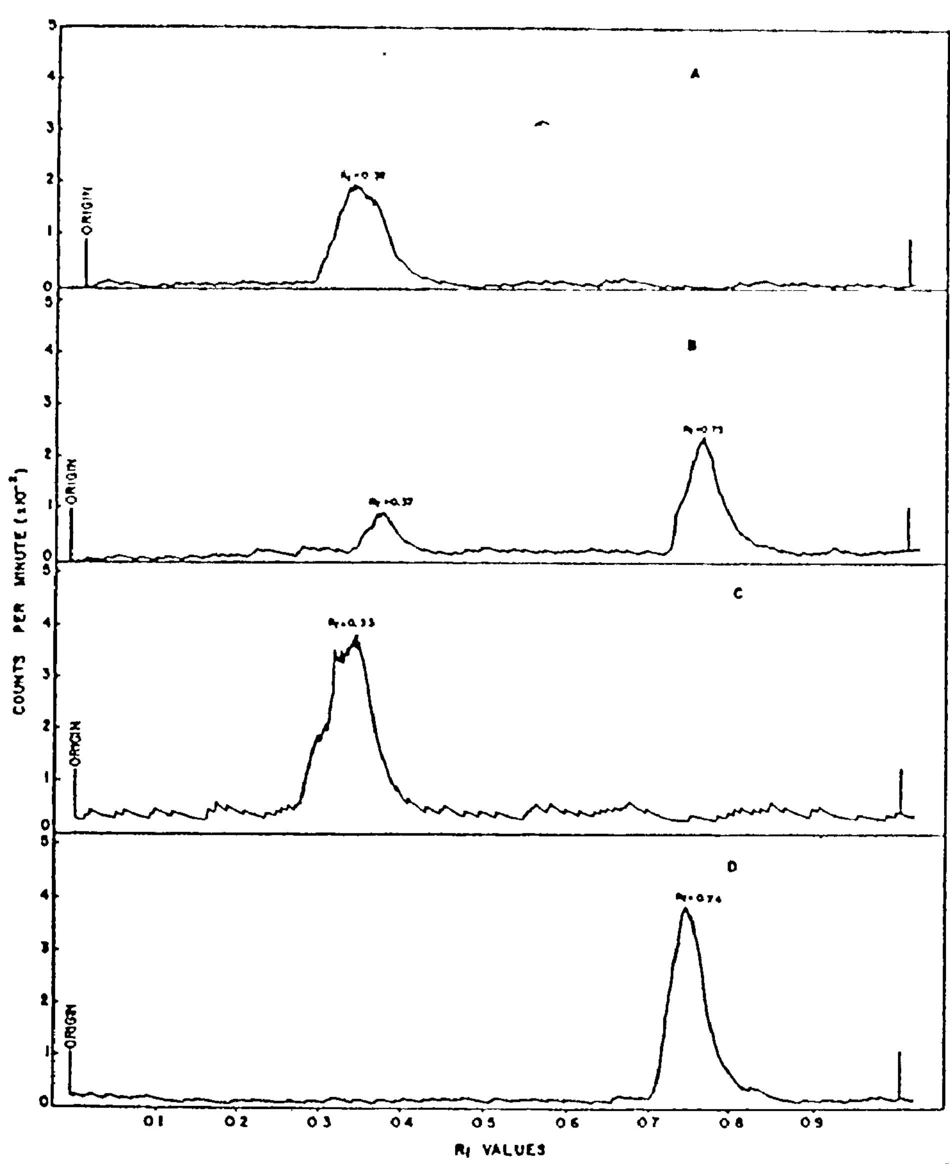


Figure 9

Figure 10. Paper chromatographic separation of fraction II

Three samples of fraction II from Figure 2 were chromatographed on paper in solvent systems A, B, and C. Authentic samples of RZ were included in all systems and were found to coincide with one of the radioactive peaks; i.e. R_f = 0.80 in A, 0.71 in B and 0.43 in C, respectively.

A : In solvent A; B : in solvent B; C : in solvent C.

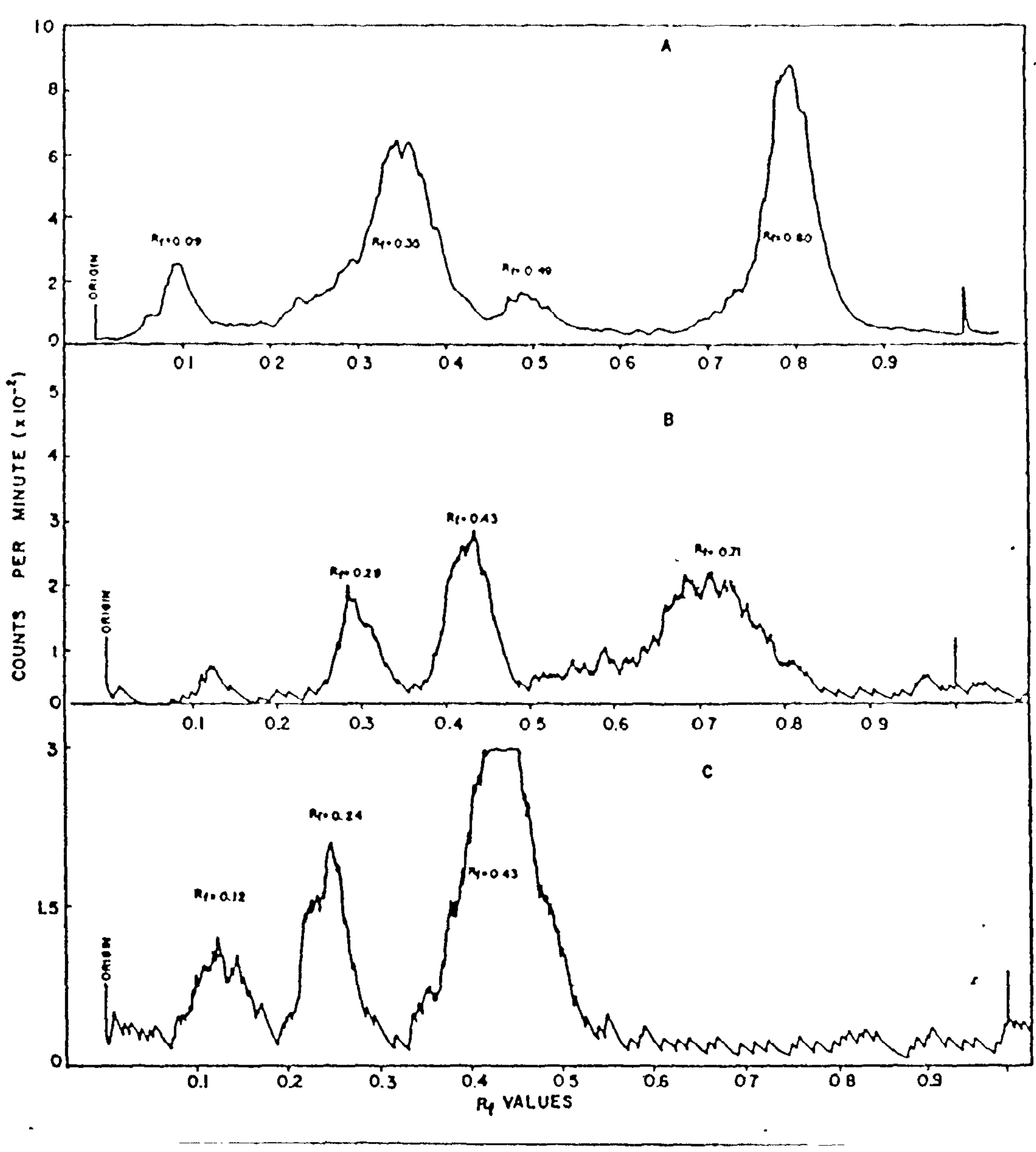


Figure 10

TABLE 2

Thin layer chromatographic separation of *cis* and *trans* - ribosyl zeatin

	R_f	Radioactivity (cpm)
Cis - RZ	0.44	62
Trans - RZ	0.30	1648

Samples of suspected RZ and a commercially-synthesized RZ, obtained from Calbiochem, were streaked on a TLC plate (Silica gel - No. 6060 with fluorescent indicator) and the chromatogram was developed in CH_3OH : $CHCl_3$ (1 : 9) solvent system. The position of the two isomers was located by UV light. Similar *cis* and *trans* positions of radioactive sample were scraped off the TLC plate into the scintillation vials, which were counted in Xylene : Triton - 114 solvent system. Duplicate samples were carried out and the mean taken for presentation.

on the same paper in all three systems. The papers were scanned with the Actigraph III and the results are shown in Figure 10.

Three radioactive peaks predominate in each system. In all cases the largest peak coincided with the authentic RZ (R_f = 0.80 in A, 0.71 in B and 0.43 in C, respectively). There were some unidentified compounds and Ado was assumed to be one of them. Confirmation studies, however, for Ado were not carried out.

(vi) Confirmation of the identity of RZ

(a) $KMnO_4$ oxidation: An eluted sample of presumed RZ from Figure 10 was treated with 0.1% $KMnO_4$ as described before. The products were separated by paper chromatography in solvent system A. A control sample and authentic samples of RZ and Ado were also run on the same paper. The results after scanning are shown in Figure 11.

The $KMnO_4$ oxidation cleaved off the side chain of the presumed RZ as shown by the fact that the majority of the radioactive product migrated in a position corresponding to authentic Ado (Figure 11B).

(b) Cytokinin oxidase treatment: Another sample of presumed RZ from Figure 10 was treated with partially purified cytokinin oxidase obtained from corn kernel extracts. A control sample without enzyme was incubated under the same conditions as the test sample. Both products were analyzed by paper chromatography in solvent system C. Authentic samples of RZ, Ado and Ade were also included on the same paper. The results are shown in Figure 12.

It can be seen that cytokinin oxidase gave a similar result as $KMnO_4$ oxidation. The majority of the radioactive material was found at the position of authentic Ado (R_f = 0.13) due to cleavage of the side

Figure 11. $KMnO_4$ oxidation of a suspected sample of RZ

A suspected sample of RZ, eluted from Figure 10, was dissolved in 0.5 ml of distilled water and the $KMnO_4$ oxidation was performed as described before. The products were chromatographed on paper in solvent system A and the paper was scanned in the Actigraph III. Authentic samples of RZ and Ado were included on the same paper. A : The suspected sample of RZ without $KMnO_4$ oxidation. Its R_f value coincided with authentic RZ; B : the sample after $KMnO_4$ oxidation. The majority of the radioactivity now corresponds to the position of Ado.

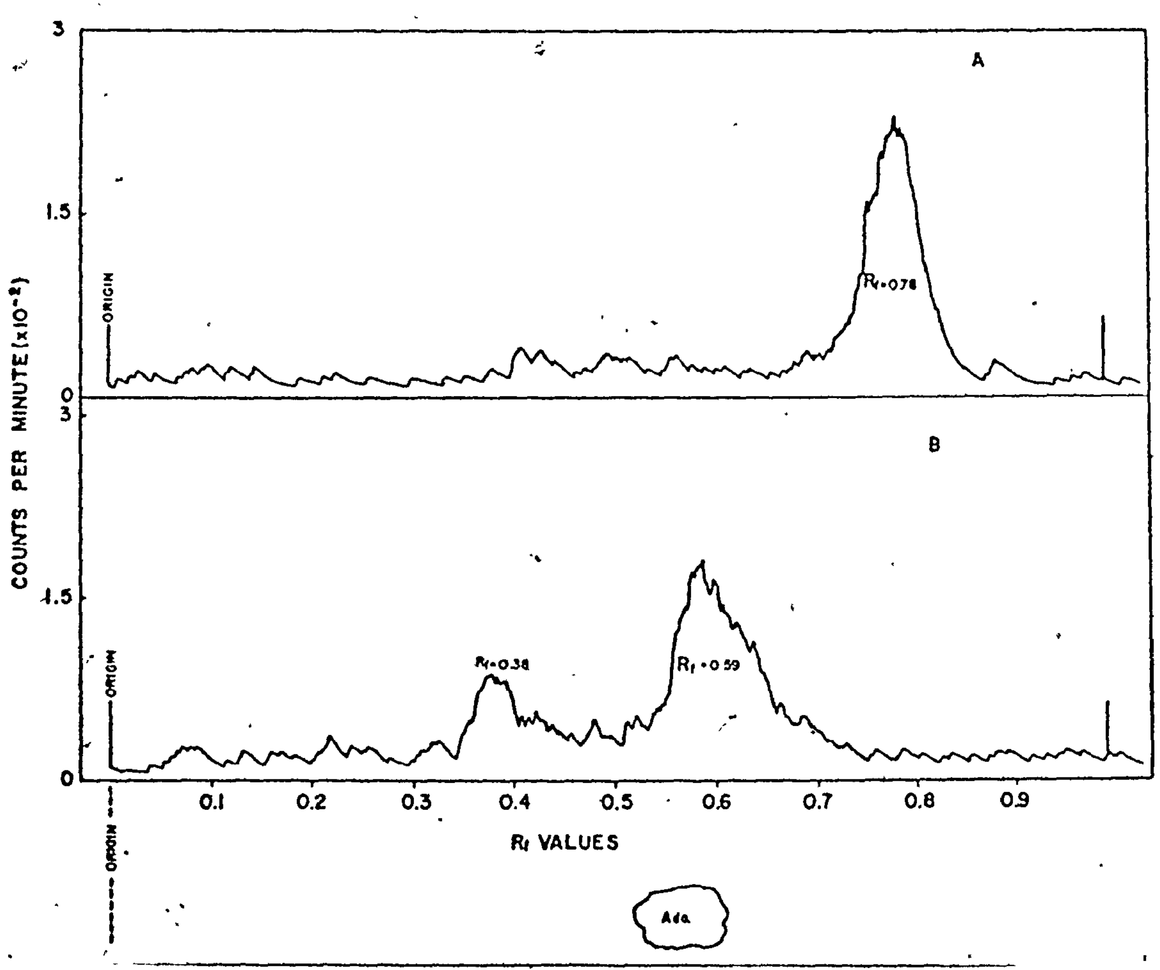


Figure 11

Figure 12. Enzymatic treatment of a suspected sample of RZ

A sample of suspected RZ, from Figure 10, was dissolved in 0.5 ml of Tris/HCl buffer (0.05 M, pH 7.5) and 0.5 ml of partially purified cytokinin oxidase from corn extract (4.6 O.D./ml) was added. The incubation was performed for 5 hours at 37°C. Another sample of suspected RZ was incubated under the same conditions without enzyme. The products were chromatographed on paper in solvent C. Authentic samples of RZ, Ado and Ade were also run on the same paper. A : The result with a suspected sample of RZ without enzyme; B : the result with the enzyme treated sample.

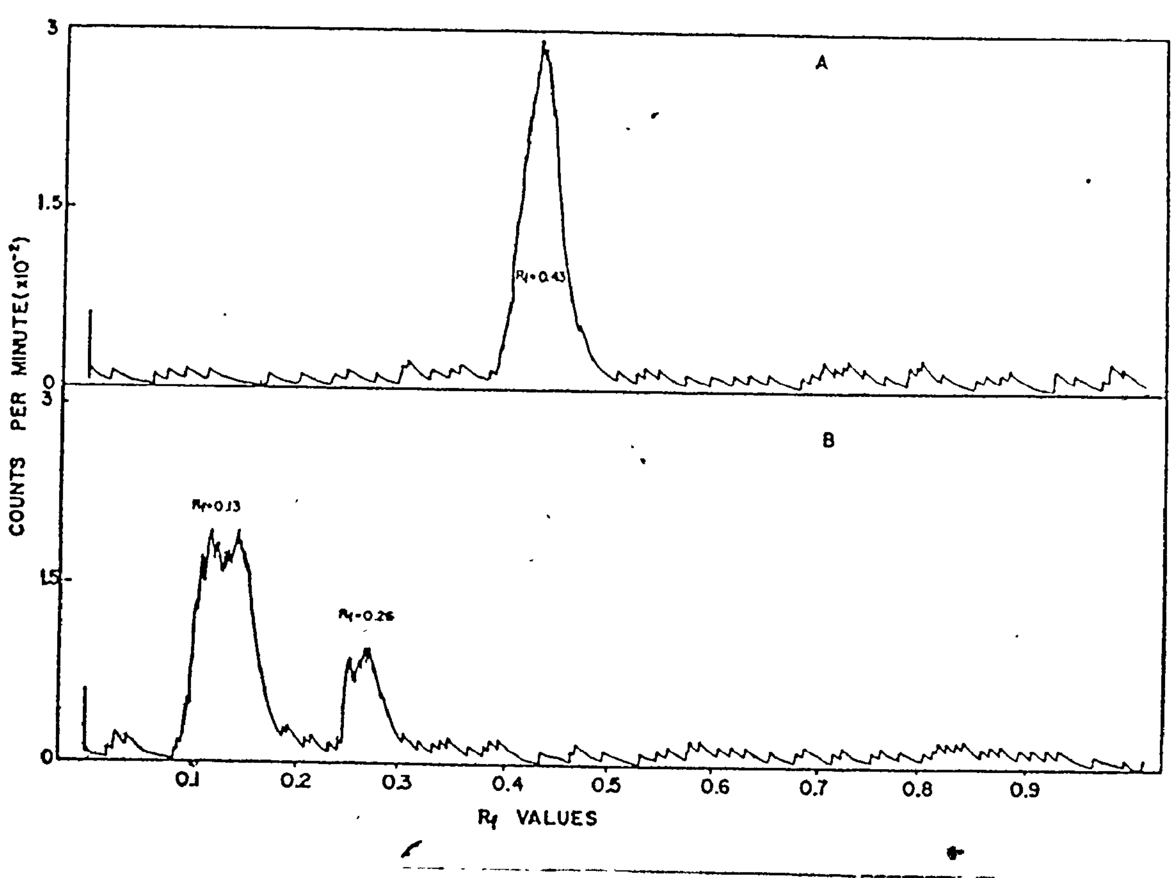


Figure 12

chain of RZ. Freshly prepared corn extracts also contained a small amount of nucleosidase activity (Whitty and Hall, 1974) and because of this enzyme, another product (R_f = 0.26) was obtained. This product coincided with authentic Ade (Figure 12B), whereas in the control sample, the compound migrated in the position of authentic RZ (R_f - 0.43) (Figure 12A).

(c) TLC: Another sample of the suspected RZ was run on a thin layer chromatogram in CH_3OH : $CHCl_3$ (1 : 9) solvent system. A sample of RZ, obtained from Calbiochem, which consisted of a mixture of *cis* and *trans*-isomers, was also run on the same TLC plate. The majority of the radioactivity coincided with the position of the *trans*-isomer position (Table 2).

These results show that the compound under investigation was *trans*-RZ.

(vii) Paper chromatographic separation of fraction III

Three samples of fraction III, Figure 2, were separated into individual compounds by paper chromatography in solvent systems C, D and E. A marker of Z was included on the same paper in each system. The paper was scanned and the results are shown in Figure 13.

In all three systems there was a single peak which migrated at the same R_f value as authentic Z (R_f = 0.52 in A, 0.61 in B and 0.53 in C). It was assumed that Ade was one of the two unidentified compounds, but confirmation studies for Ade were not carried out.

(viii) Conformation studies of the identity of Z

Similar experiments to those performed in the confirmation studies of RZ were applied to the suspected sample of Z from Figure 13. The results

Figure 13. Paper chromatographic separation of fraction III

Three samples of fraction III from Figure 2 were further fractionated by paper chromatography in three solvent systems. An authentic sample of Z was included on the same paper where it coincided with the largest radioactive peak in all systems; i.e. R_f = 0.52 in A, 0.61 in B and 0.53 in C, respectively. A : In solvent C; B : in solvent D; C : in solvent E.

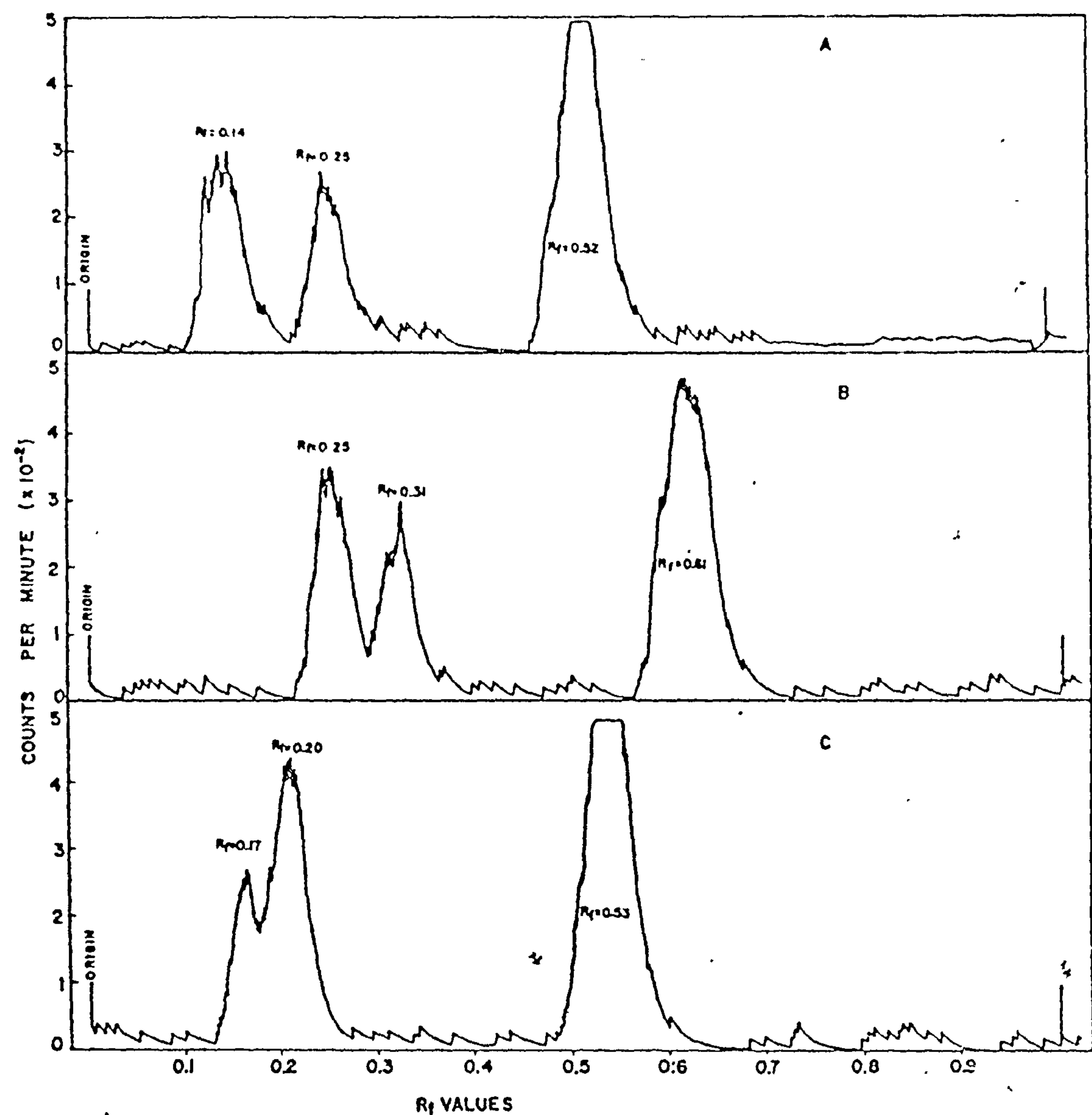


Figure 13

are shown in Figures 14 and 15 and in Table 3, and they indicate that the compound under investigation was *trans*-zeatin.

(ix) Paper chromatographic separation of fraction IV

Three samples of fraction IV from Figure 2 were resolved by paper chromatography in solvent systems A, B and E. A marker sample of i^6Ado was also run on the same paper in each system. The papers were scanned and the results are shown in Figure 16.

In all cases a single radioactive peak appeared which coincided with authentic i^6Ado. Confirmation of the identity of i^6Ado was carried out as follows.

(x) Confirmation studies of the identity of i^6Ado

(a) $KMnO_4$ oxidation: Two samples of suspected i^6Ado from Figure 16 were treated with 0.1% $KMnO_4$ as described previously. The products were separated by paper chromatography in two different solvent systems. An untreated sample of suspected i^6Ado and authentic samples of i^6Ado and Ado were included on the same paper. The developed paper was scanned and the results are shown in Figure 17 (solvent A) and Figure 18 (solvent D).

In both cases the $KMnO_4$ treated samples migrated differently from that of controls. The sample which had undergone $KMnO_4$ oxidation migrated at R_f values of 0.50 (Figure 17B) and 0.19 (Figure 18B), both of which coincide with that of authentic Ado. In the control samples, the radioactive peaks were found at the position of authentic i^6Ado in both solvent systems (R_f = 0.89 in Figure 17A; 0.81 in Figure 18A).

(b) Adenosine aminohydrolase treatment: Another sample of suspected i^6Ado was treated with adenosine aminohydrolase and the products were

Figure 14. $KMnO_4$ oxidation of a suspected sample of Z

A sample of suspected Z from Figure 13 was dissolved in 0.5 ml of distilled water and a $KMnO_4$ oxidation was performed as described before. The products were chromatographed on paper in solvent system E and the developed paper was scanned in the Actigraph III. Authentic samples of Z and Ade were run on the same paper. A : The untreated sample, which migrated with authentic Z; B : after $KMnO_4$ oxidation. The sample migrated with authentic Ade.

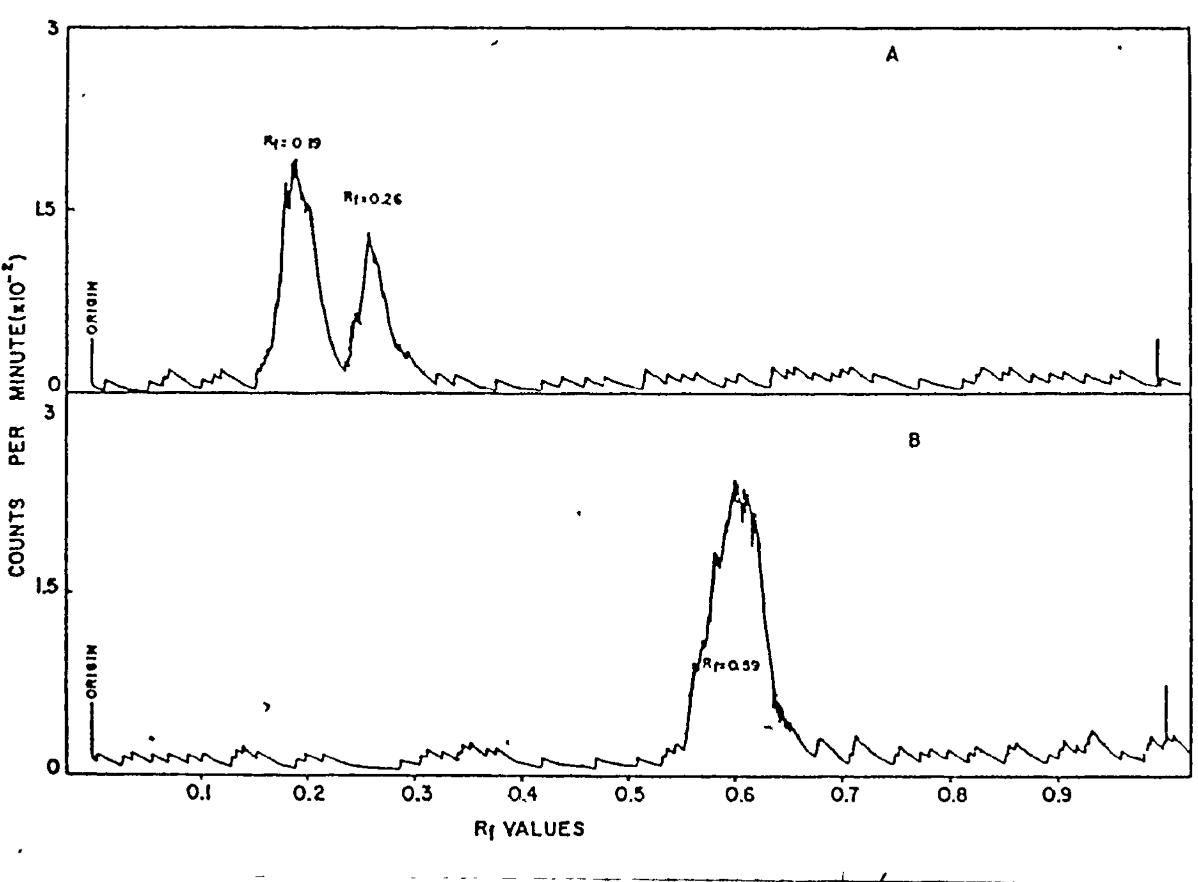


Figure 14

Figure 15. Enzymatic treatment of putative Z

A sample of suspected Z, eluted from Figure 13, in 0.5 ml of Tris/HCl buffer (0.05 M, pH 7.5) was treated with 0.5 ml of partially purified cytokinin oxidase from corn extracts (4.6 O.D./ml). The incubation was performed for 5 hours at 37°C. Another sample of suspected Z was incubated under the same conditions, without enzyme. The products were analyzed by paper chromatography in solvent system C. A : Sample incubated without enzyme; B : sample incubated with cytokinin oxidase; it coincided with authentic Ade, which was also run on the same paper.

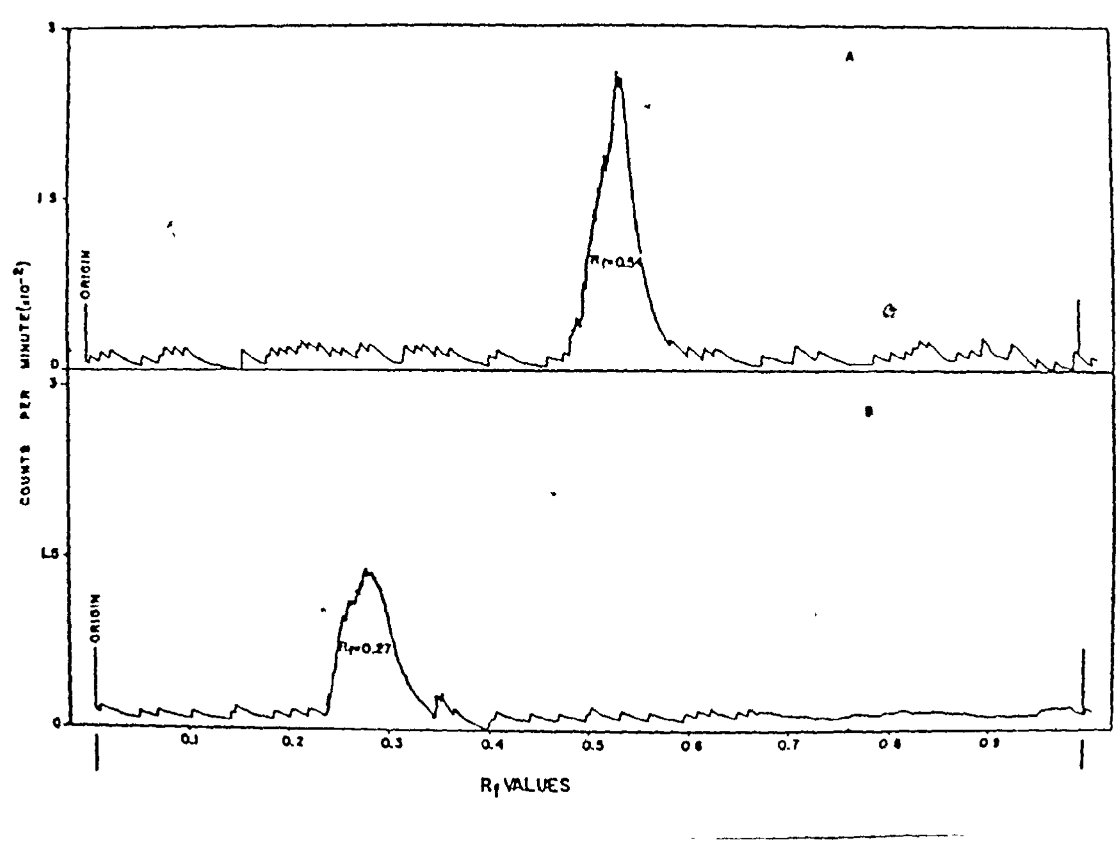


Figure 15

TABLE 3

Thin layer chromatographic separation of

cis and *trans* - zeatin

	$\underline{R}_f$	Radioactivity (cpm)
Cis - zeatin	-	-
Trans - zeatin	0.35	1726

Samples of suspected Z and commercial Z, obtained from Calbiochem, were streaked on a TLC plate (Silica gel - No. 6060 with fluorescent indicator) and the chromatogram was developed in CH_3OH : $CHCl_3$ (1 : 9) solvent system. The position of the two isomers were located by UV light. Only *trans* - Z was obtained from the reference sample. The corresponding position of the radioactive sample was scraped off the TLC plate into a scintillation vial. It was then counted in the Xylene : Triton - 114 solvent system. Duplicate samples were analyzed and the average of the two was used as the recorded value.

Figure 16. Paper chromatographic separation of fraction IV.

Three samples of fraction IV from Figure 2 were further analyzed by paper chromatography in three solvent systems. An authentic sample of i^6Ado, run on the same paper coincided with the single peak in all systems.
A : In solvent A; B : in solvent D and C : in solvent E.

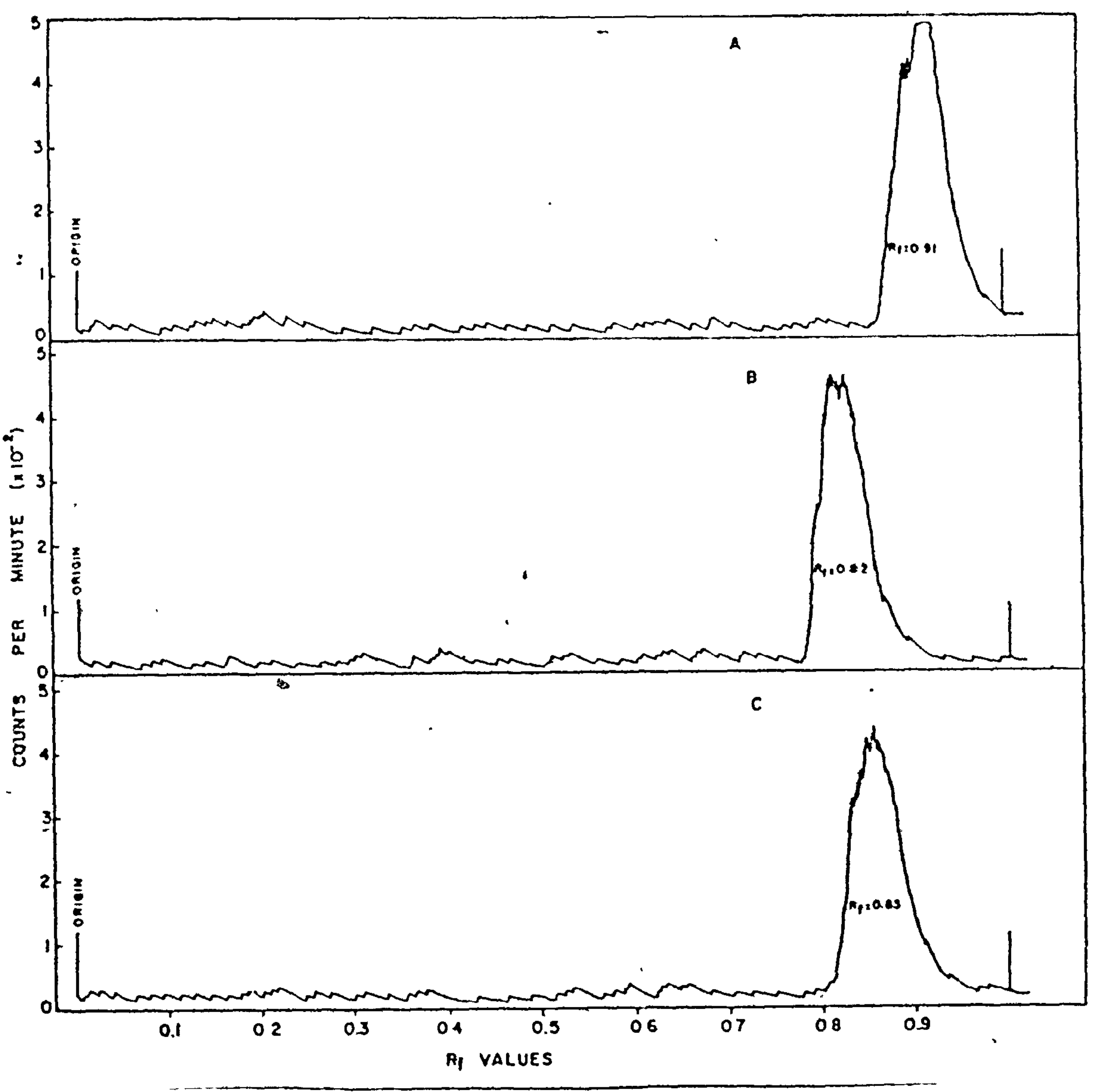


Figure 16

Figure 17. $KMnO_4$ oxidation of putative i^6Ado

A sample of suspected i^6Ado, eluted from Figure 16, was dissolved in 0.5 ml of distilled water and the $KMnO_4$ oxidation was performed as described before. The products were chromatographed on paper in solvent A and the paper was scanned in the Actigraph III. Authentic samples of i^6Ado and Ado were also run on the same paper. A : Suspected i^6Ado without $KMnO_4$ treatment; its position coincided with authentic i^6Ado; B : the sample after $KMnO_4$ oxidation; the majority of the radioactivity has shifted to the position of authentic Ado (R_f = 0.50).

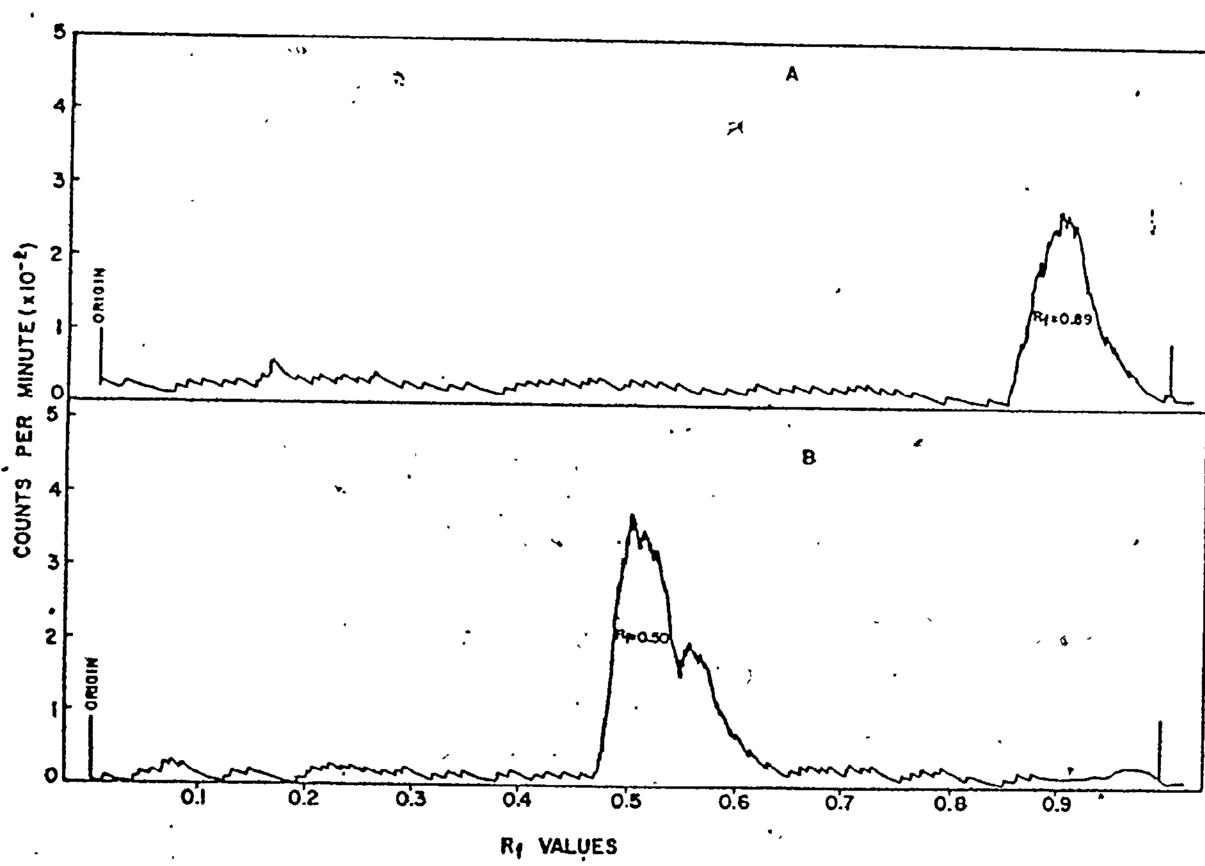


Figure 17

Figure 18. $KMnO_4$ oxidation of putative i^6Ado

The samples described in Figure 17, chromatographed in solvent D.
A : The untreated control; B : the sample after $KMnO_4$ oxidation.

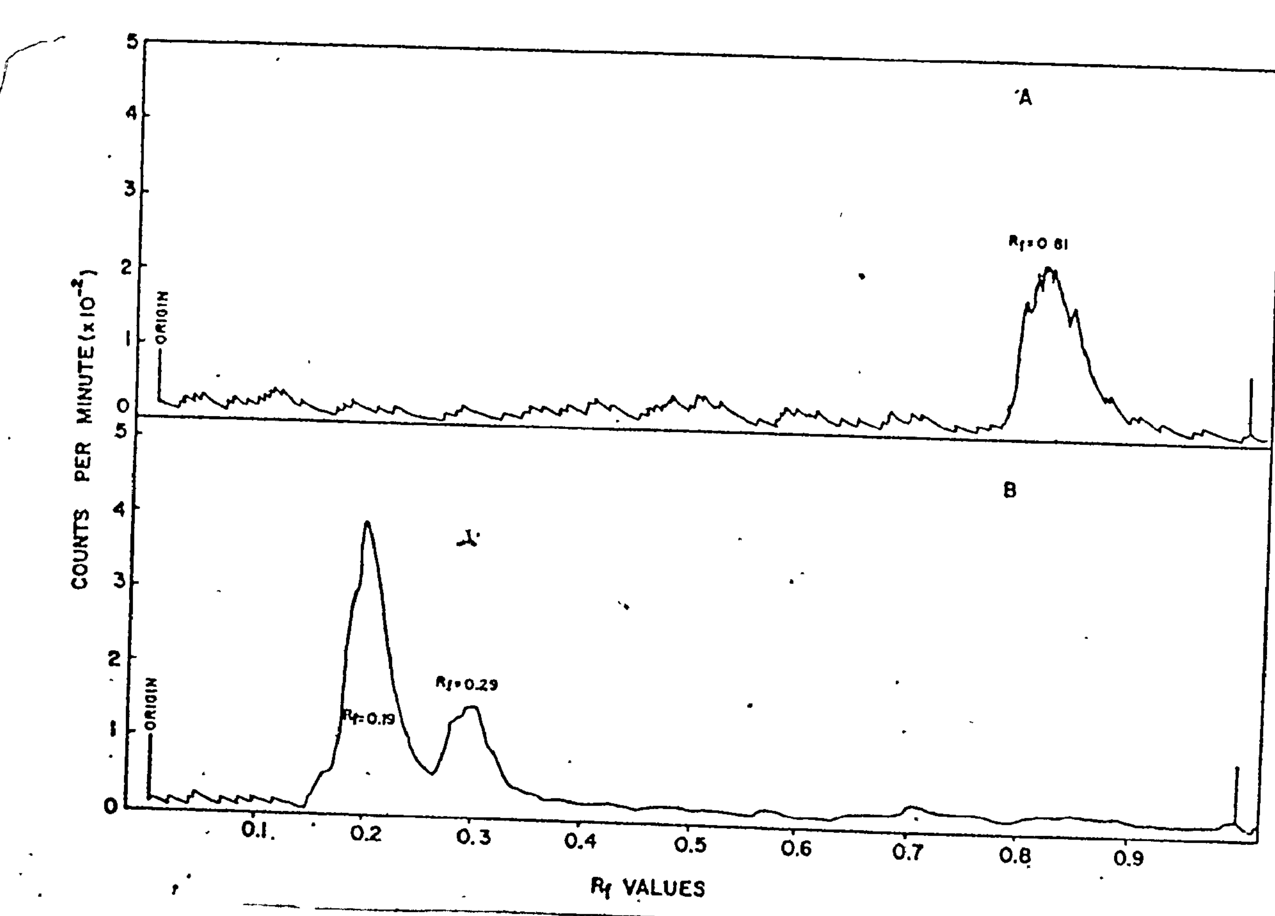


Figure 18

analyzed by paper chromatography in solvent system A. A control sample without enzyme was incubated under the same conditions as the test sample. Authentic samples of inosine and i^6Ado were also run on the same paper. The developed paper was scanned in an Actigraph III and the results are shown in Figure 19.

The enzyme treated sample migrated in the position of authentic inosine (R_f = 0.38) (Figure 19B);the untreated control migrated to the position of authentic i^6Ado (R_f = 0.91 in Figure 19A).

Figure 20 shows the results of a similar experiment in which the paper was developed in solvent system E.

In summary, these experiments demonstrate that the compound under investigation is i^6Ado.

(xi) Paper chromatographic separation of fraction V

Three samples of fraction V from Figure 2 were further separated by paper chromatography in solvent systems C, D and E. An authentic sample of i^6Ade was also run in each system. The paper was scanned and the results are shown in Figure 21.

A single radioactive peak was obtained which coincides with authentic i^6Ado in all systems. For confirmation, the following experiments were carried out.

(xii) Confirmation studies of the identity of i^6Ade

(a) $KMnO_4$ oxidation: A sample of the suspected i^6Ade from Figure 2 was treated with 0.1% $KMnO_4$. The products were analyzed by paper chromatography in solvent system E. Another sample of suspected i^6Ade which was not treated with $KMnO_4$ and authentic samples of i^6Ade and Ade were also run on the same paper. The paper was scanned in the Actigraph

Figure 19. Enzymatic treatment of putative i^6Ado

A sample of suspected i^6Ado from Figure 16 in 0.5 ml of Tris/HCl buffer (0.05 M, pH 7.5) containing 0.005 M $MgCl_2$ and 0.01 M mercaptoethanol was incubated with adenosine aminohydrolase (2 I.U.) for 5 hours at 37°C. Another sample of suspected i^6Ado was incubated under the same conditions without enzyme. The products were chromatographed on paper in solvent system A. Marker samples of inosine and i^6Ado were also run on the same paper. A : the sample incubated without enzyme; this R_f coincides with authentic i^6Ado; B : the sample treated with adenosine aminohydrolase; this R_f coincides with authentic inosine.

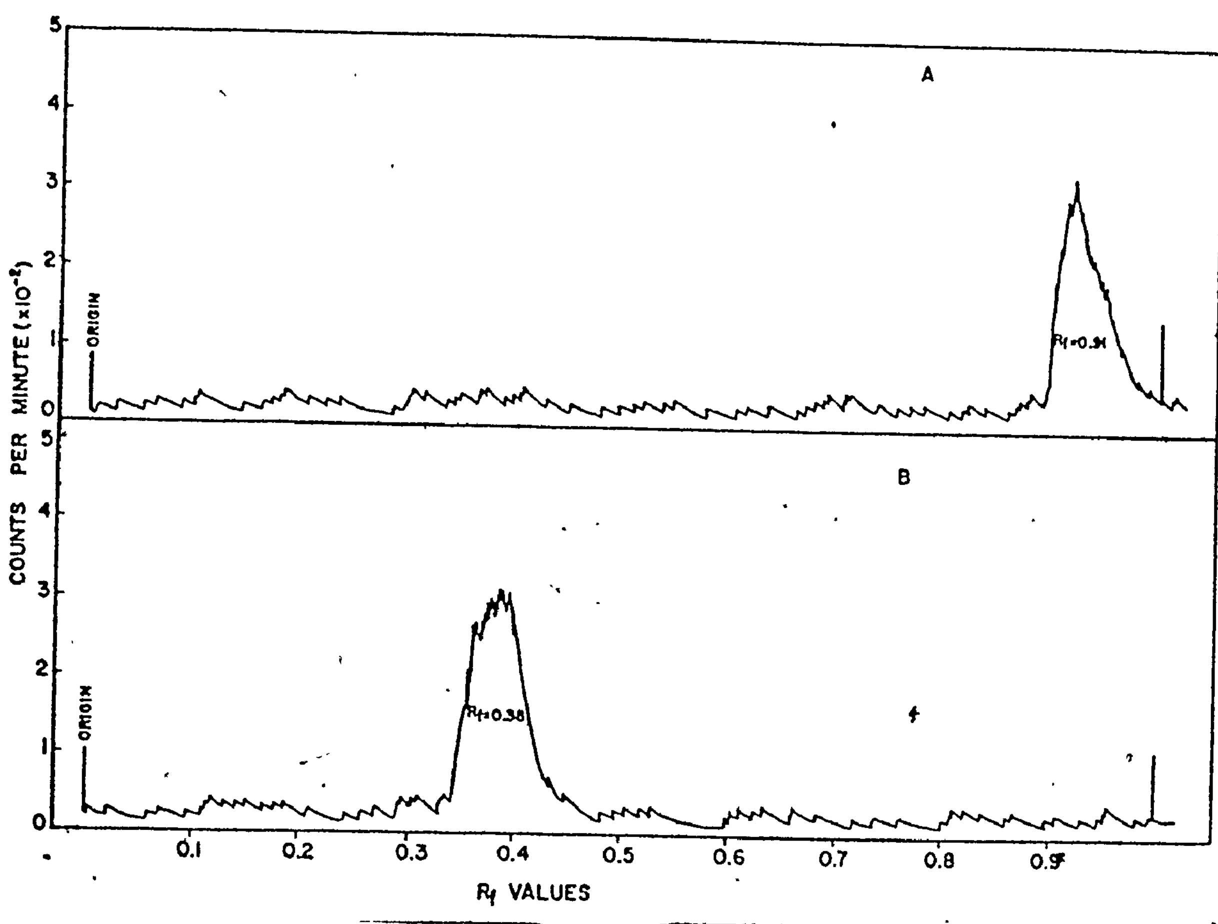


Figure 19

Figure 20. Enzymatic treatment of putative i^6Ado

The samples described in Figure 19, chromatographed in solvent E.

A : The untreated control; B : the adenosine aminohydrolase treated sample.

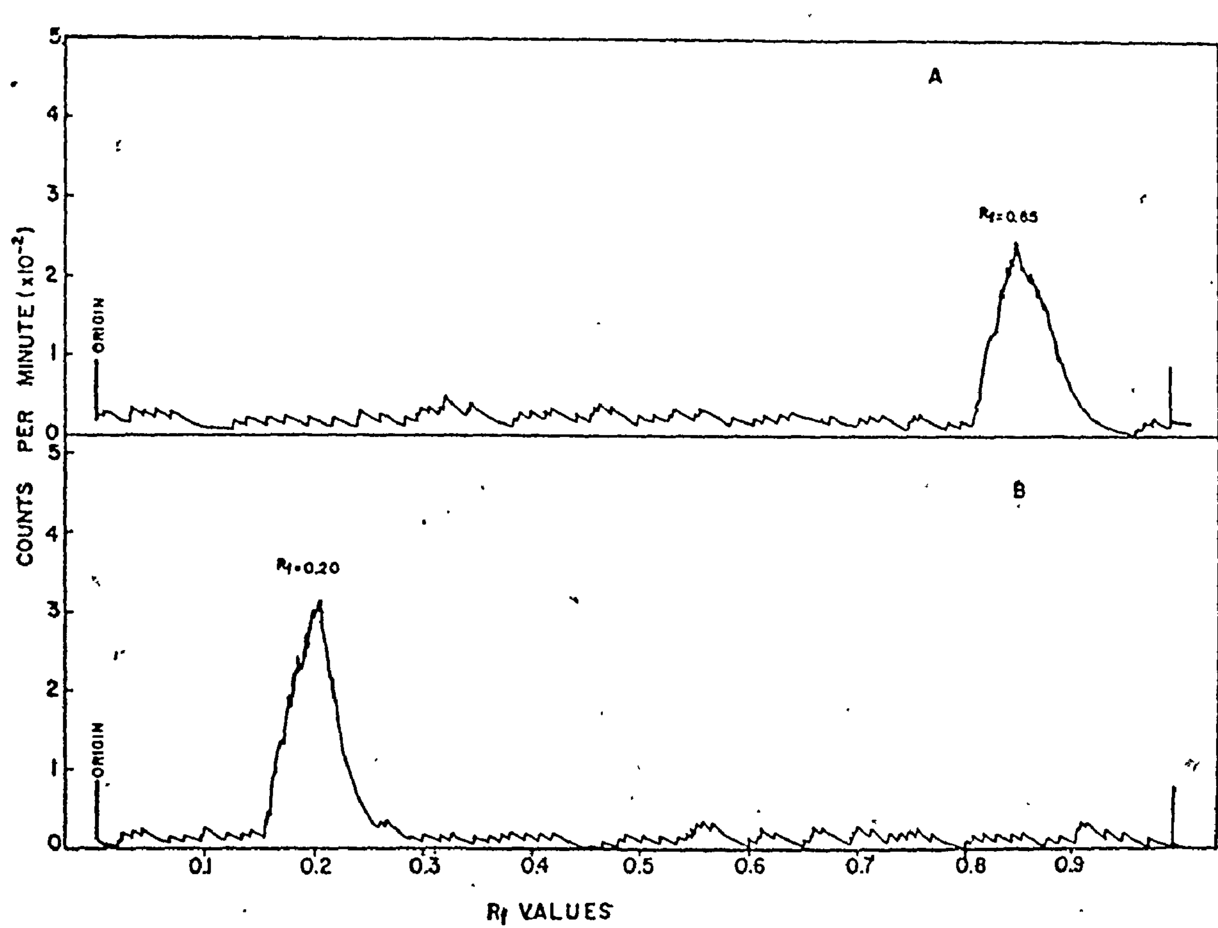


Figure 20

Figure 21. Paper chromatographic separation of fraction V

Three samples of fraction V from Figure 2 were further analyzed by paper chromatography in three solvent systems. The papers were scanned in the Actigraph III. Authentic samples of i^6Ade were run on the same paper and found to coincide with the single radioactive peak in all systems.
A : Solvent C; B : solvent D and C : solvent E.

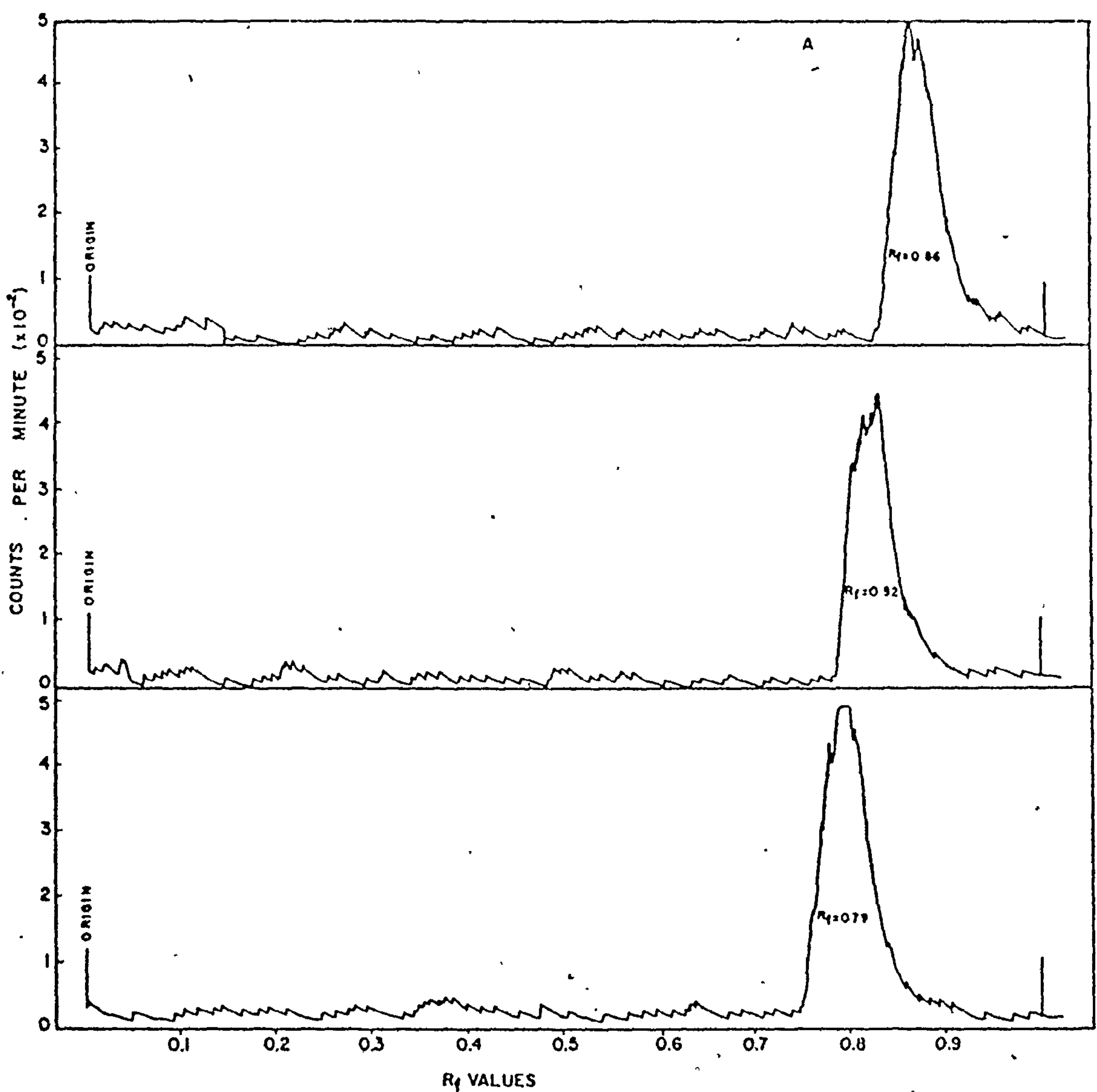


Figure 21

III and the results are shown in Figure 22.

The $KMnO_4^-$ treated sample migrated in a position corresponding to authentic Ade (R_f = 0.29 in Figure 22B). However, the untreated control sample remained in the position of authentic i^6Ade (R_f = 0.80 in Figure 22A).

(b) Cytokinin oxidase treatment: Another sample of suspected i^6Ade was treated with cytokinin oxidase from corn extracts and the products were analyzed by paper chromatography in solvent system D. A control sample without enzyme treatment was incubated under the same conditions as the test sample. The results are shown in Figure 23.

The R_f value of the enzyme treated sample was found to be 0.20, which coincides with authentic Ade, whereas the untreated control gave an R_f value of 0.82, which corresponds to authentic i^6Ade.

From these experiments it was concluded that the compound under investigation was i^6Ade.

(xiii) Summary of identification experiments

These experiments provide evidence that i^6Ado was metabolized into i^6Ade, RZ, Z and their respective 5'-nucleotides in KX tissue. Similar results were also obtained with O-1 tissue. When the incubation medium was changed from phosphate buffer to the basal media and the incubation temperature reduced from 37°C to 27°C, a similar pattern of products, but lesser in amount, was also obtained.

(xiv) Control experiments

In order to prove that these compounds were the metabolites of i^6Ado formed by the experimental tissues, a control sample of [8-^{14}C]i^6Ado was incubated in phosphate buffer as well as in basal medium lacking the agar, under the same conditions without any tissue. It was analyzed on a LH-20

Figure 22. $KMnO_4$ oxidation of putative i^6Ade

A sample of suspected i^6Ade from Figure 21 was dissolved in 0.5 ml of distilled water and the $KMnO^4$ oxidation was performed as described before. The products were chromatographed in solvent system E and the developed paper was scanned in the Actigraph III. Marker samples of i^6Ade and Ade were run on the same paper.

A : The control sample, which coincides with authentic i^6Ade;

B : the sample after $KMnO_4$ oxidation, which coincides to the position of authentic Ade.

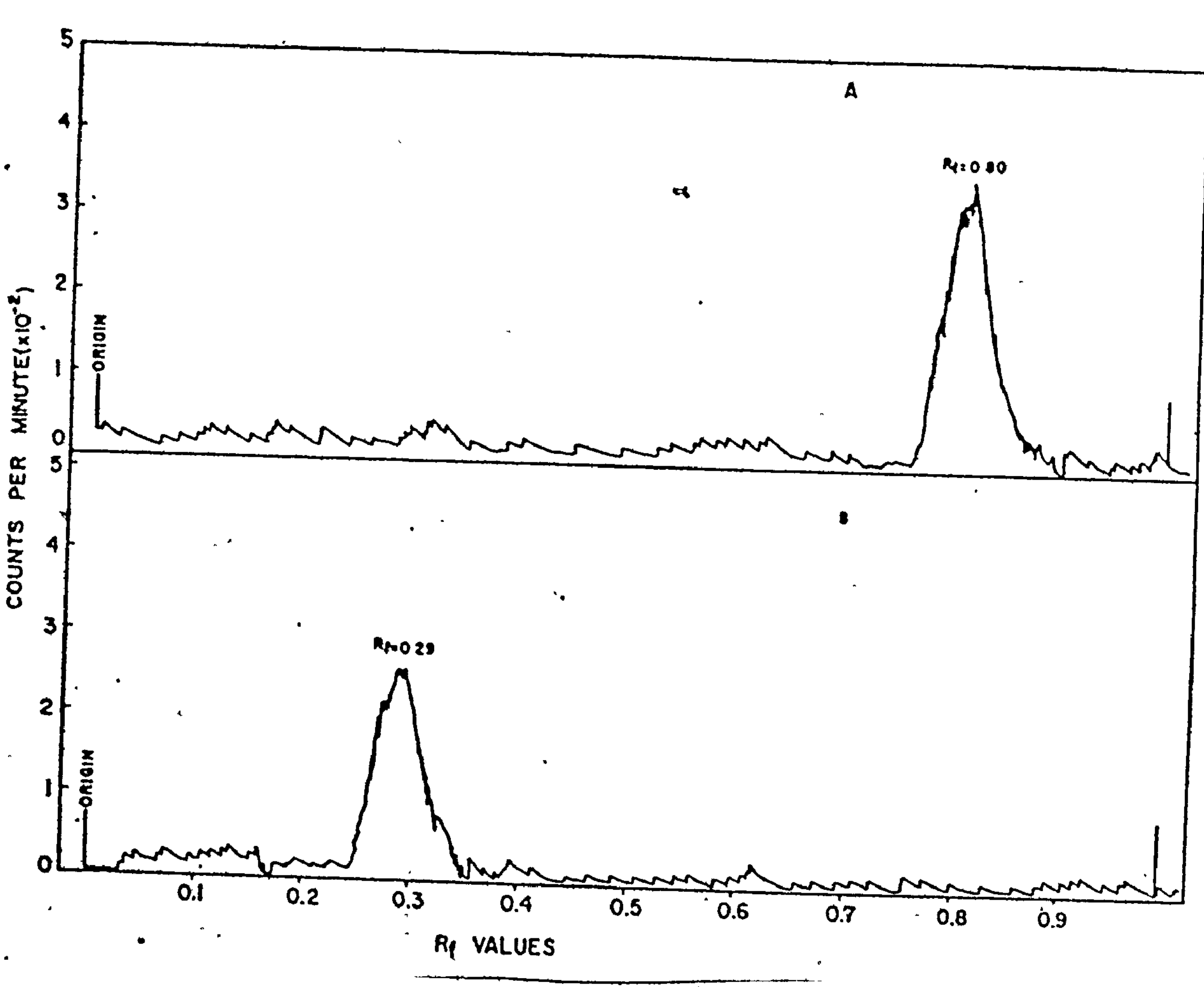


Figure 22

Figure 23. Enzymatic treatment of putative i^6Ade

A sample of suspected i^6Ade from Figure 21 was dissolved in 0.5 ml of Tris/HCl buffer (0.05 M, pH 7.5) and 0.5 ml of partially purified cytokinin oxidase from corn extracts (4.6 O.D./ml) was added. The incubation was performed for 5 hours at 37°C. A control sample was incubated under the same conditions without enzyme extracts. The products were chromatographed on paper in solvent system D. Authentic samples of i^6Ade and Ade were also run on the same paper. A : The presumed sample without enzyme treatment; the R_f value coincides with that of authentic i^6Ade; B : the sample treated with cytokinin oxidase; the R_f value of the radioactive peak was located at the position of authentic Ade.

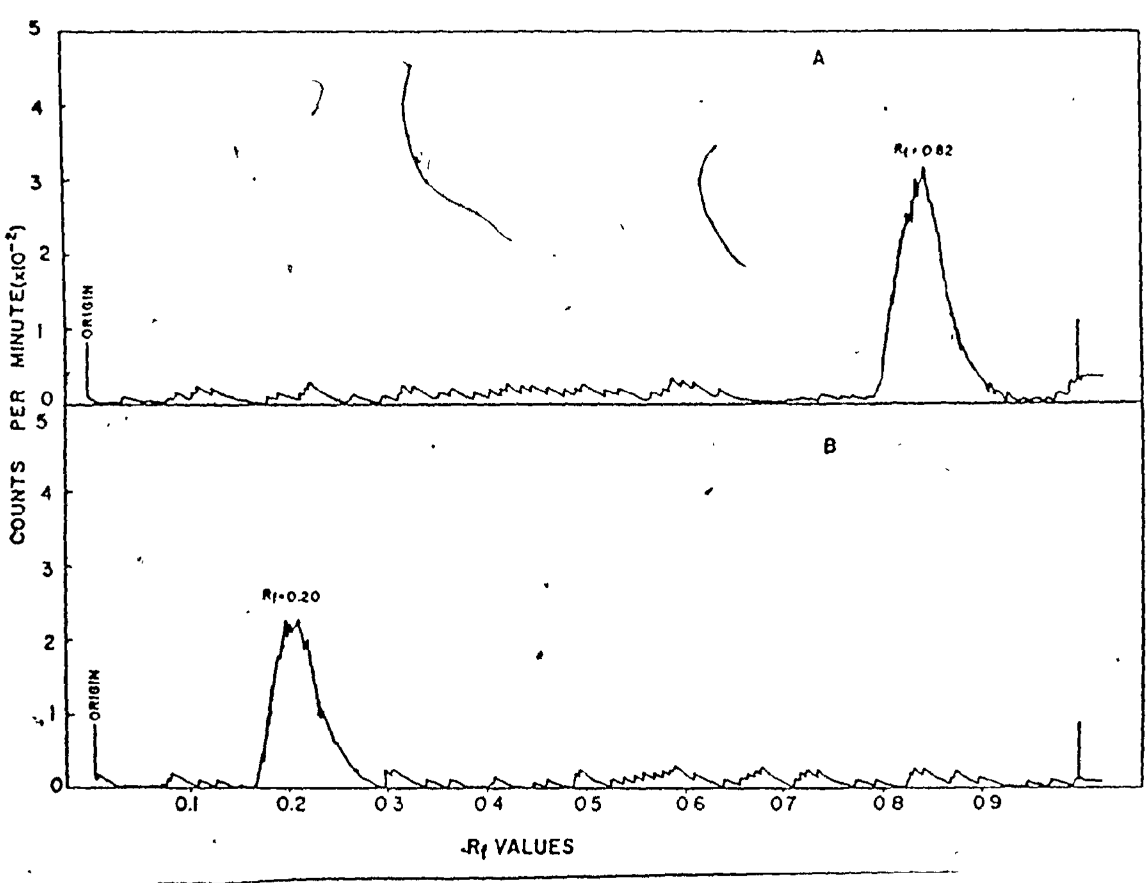


Figure 23

column and the results are shown in Figure 24.

It can be seen that except for a small peak at the Ado fraction there were no other compounds in addition to [^{14}C] i^{6}Ado, i.e. fraction 76 to 90. The small peak was identified as Ado by paper chromatography in different solvent systems as shown above. This result demonstrates that only a small amount (about 1.0% of the added [^{14}C] i^{6}Ado) of i^{6}Ado spontaneously degraded to Ado under these experimental conditions.

Another control experiment was performed in order to find out whether or not any metabolites could have leaked out of the tissue during the incubation period. The incubation medium and the washings were collected after the tissue was incubated with measured amounts of [^{14}C] i^{6}Ado in phosphate buffer or in its own basal medium. The combined solution was analyzed by LH-20 column fractionation.

Figure 25 shows the results of this study. A large peak, at the i^{6}Ado position, and a small peak, at the Ado position, were obtained. The results are quite similar to those shown in Figure 24. The small peak was identified as Ado by paper chromatography in different solvent systems as shown above. Therefore, it appears that no labelled metabolites leaked out of the tissue during the incubation. The small amount of Ado was the result of spontaneous degradation of i^{6}Ado under these experimental conditions, as was obtained in the previous experiment.

(2) Quantitative Studies

Once the metabolic products were established, the metabolism of i^{6}Ado was studied quantitatively in both the KX and O - 1 tissues. The quantitative measurements were undertaken in order to make a comparative study of i^{6}Ado metabolism in each of these cell lines. In this part of

Figure 24. Incubation of [8-^{14}C] i^6Ado with no tissue

About 6.0 x 10^5 cpm of [^{14}C] i^6Ado was incubated in a KX-basal medium, minus the agar, under sterile conditions for 8 hours at 27°C without the tissue. The incubation medium was evaporated under reduced pressure, redissolved in 35% ethanol and the product was analyzed by passing it through a LH-20 column. Fractions of 10 ml were collected and 0.5 ml from each fraction was taken for scintillation counting. Duplicate samples were run for each determination.

A similar pattern was obtained when the labelled i^6Ado was incubated in phosphate buffer at 37°C.

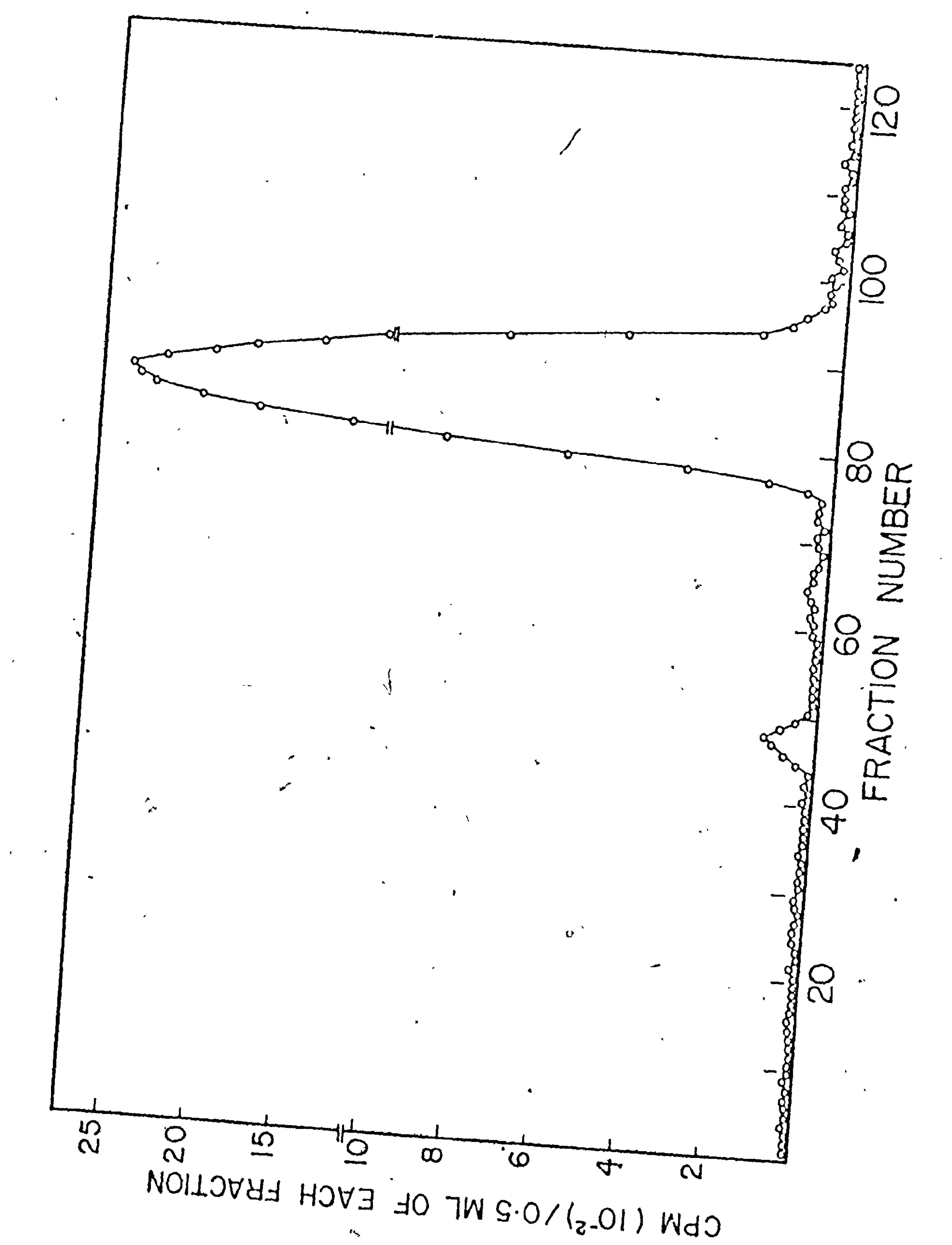


Figure 24

Figure 25. Analysis of the basal medium after the incubation of [^{14}C] i^6Ado with KX tissue

About 6.0 x 10^5 cpm of [^{14}C] i^6Ado was incubated with KX tissue in phosphate buffer for 8 hours at 37°C. The tissue was filtered and washed with phosphate buffer containing unlabelled i^6Ado. The incubation medium and the washing solution were combined and evaporated under reduced pressure. The residue was redissolved in 35% ethanol and analyzed by LH-20 column fractionation. Fractions of 10 ml were collected and 0.5 ml from each fraction was taken for scintillation counting. Duplicate samples were run for each determination and the experiment was repeated twice. A similar pattern was obtained with the 0-1 tissue.

When the experiments were repeated with their basal media, similar patterns were also obtained.

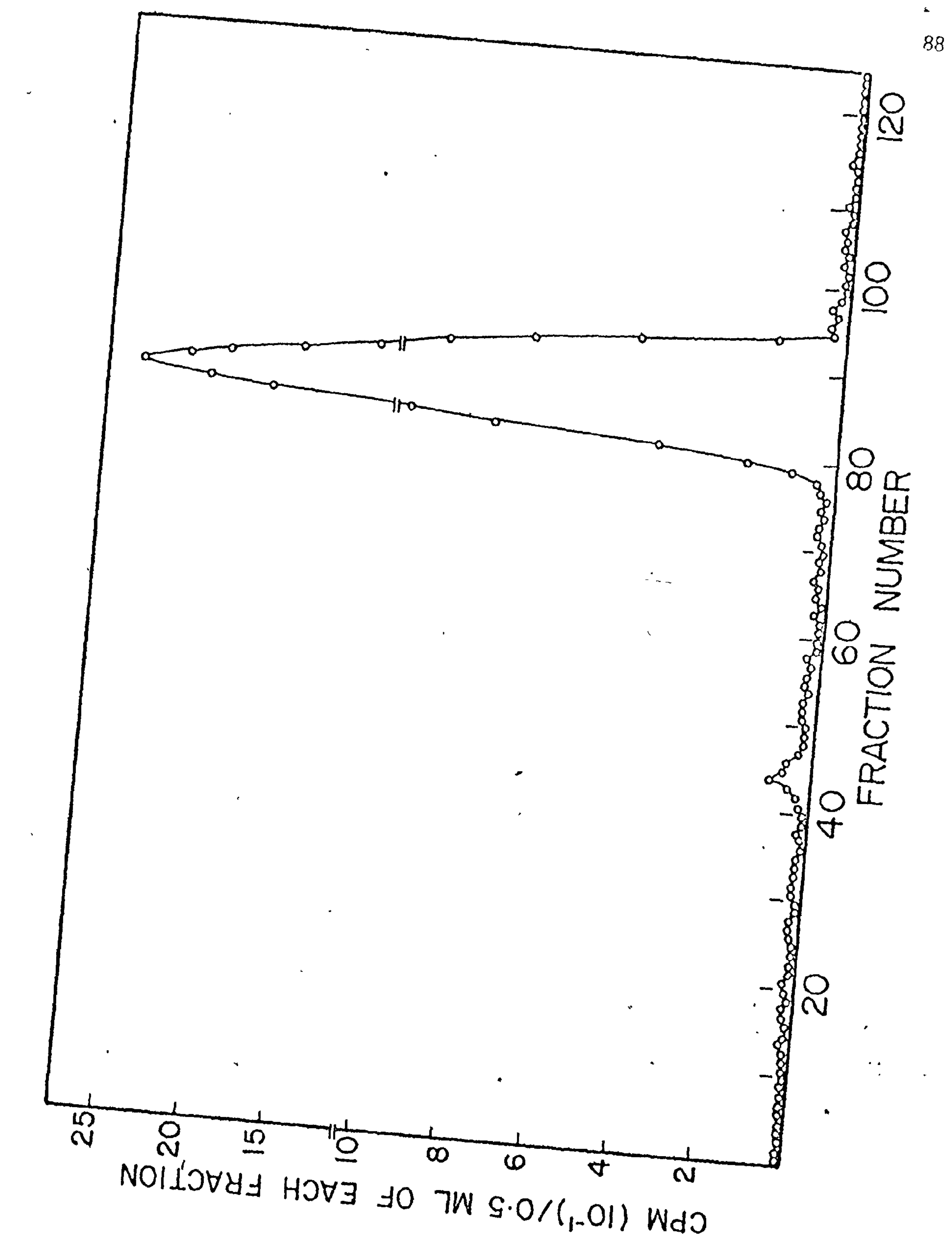


Figure 25

the study, not only the ethanol soluble fraction but also the ethanol insoluble residue and the incubation medium plus the washing solution were analyzed. Calculation of the percent recovery of the added radioactivity for each experiment was based on the total amount obtained from the above mentioned three fractions. It was found that recovery of radioactive material was between 86% and 94%.

(i) Effect of a preincubation period on the uptake of [8-^{14}C] i^6Ado in plant tissue

Because the tissue was grown on a semi-solid medium (agar) and the actual experiments were carried out in a liquid medium, the adaptability of the tissue to the new environmental conditions (i.e. the incubation medium) was studied. As described previously, the incubation medium was changed from buffer A to buffer B. Buffer B consisted of the basal medium of the tissues without the agar and with the appropriate plant hormones depending upon the experimental conditions.

It can be seen in Figures 26 and 27 that the rate of uptake of [^{14}C] i^6Ado by both KX and 0 - 1 tissues did not increase after four and two hours of preincubation periods, respectively. These data imply that the tissue could adapt to the new environment within four hours of preincubation, which is why in later experiments the preincubation period was fixed at four hours. It was assumed that within this period the uptake of [^{14}C] i^6Ado by both tissues reached a constant rate under the conditions of the experiment.

(ii) Studies on the uptake of [8-^{14}C] i^6Ado by plant tissue

A comparative study between the uptake of [^{14}C] i^6Ado by KX and 0 - 1 tissues was carried out. The results are shown in Figure 28. The

Figure 26. Effect of preincubation period on the uptake of [8-^{14}C] i^6Ado in KX tissue

KX tissue (0.5 gm) in 1.0 ml of the basal medium,except the agar and the kinetin, was incubated for 1, 2, 4, 8 and 16 hours at 27°C. About 3.0 x 10^5 dpm of [^{14}C] i^6Ado was added at the end of each of the preincubation periods and incubation was continued for another 2 hours for each determination. These procedures were done under sterile conditions.

The tissue was filtered through Millipore filters and washed thoroughly with 50 μM unlabelled i^6Ado solution. It was suspended into 1.0 ml of 2.0 M KOH in a conical centrifuge tube and hydrolyzed for 24 hours at 50°C. Two ml of 2.0 M HCl was added and the hydrolysis was continued for another 24 hours at 50°C. It was centrifuged and 0.5 ml of the supernatant was taken for radioactivity determination. Duplicate samples were run for each period. The experiment was repeated and a similar pattern of results were obtained.

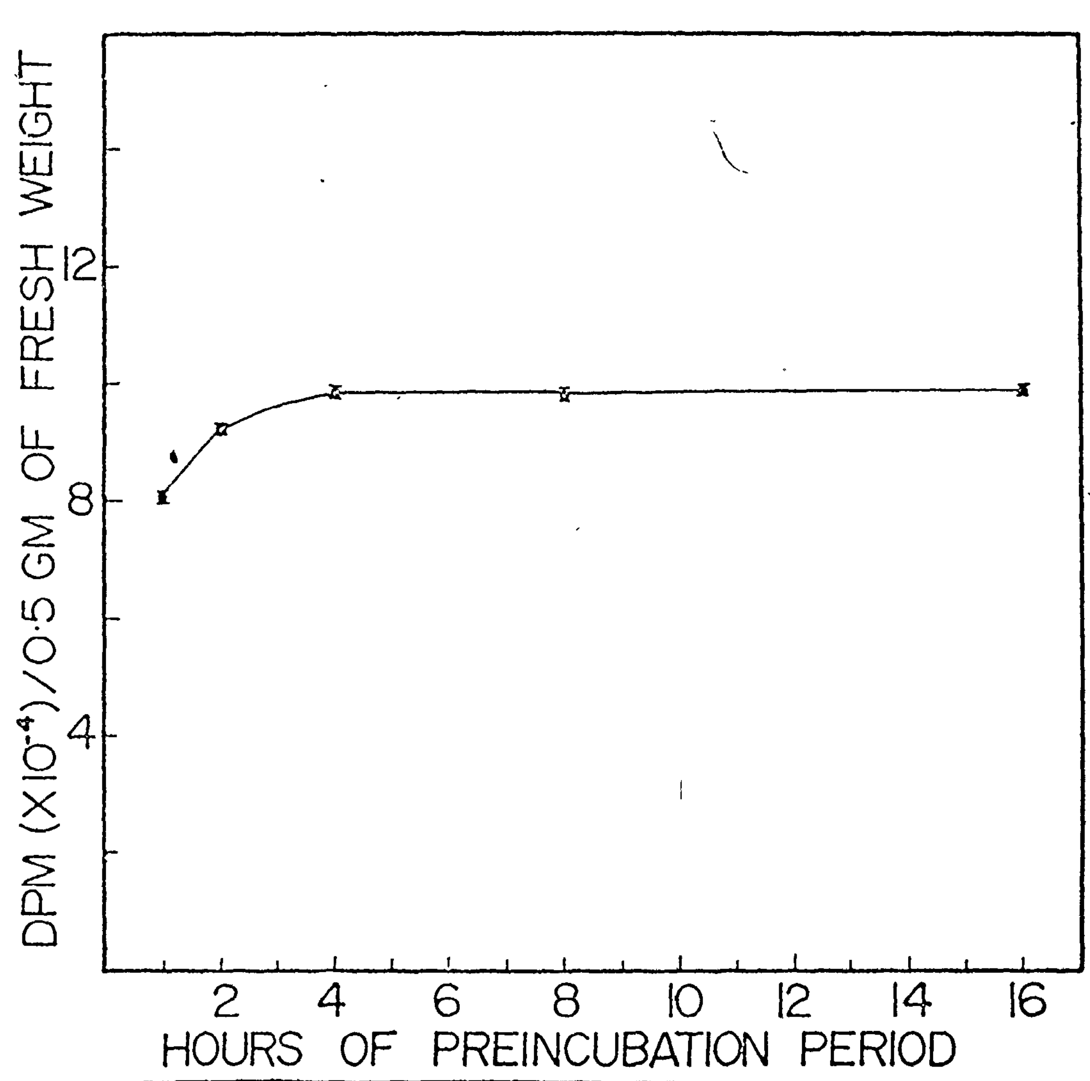


Figure 26

Figure 27. Effect of preincubation period on the uptake of [8-^{14}C]i^{6}Ado in 0-1 tissue

The experimental conditions were the same as those described for KX tissue (Figure 26).

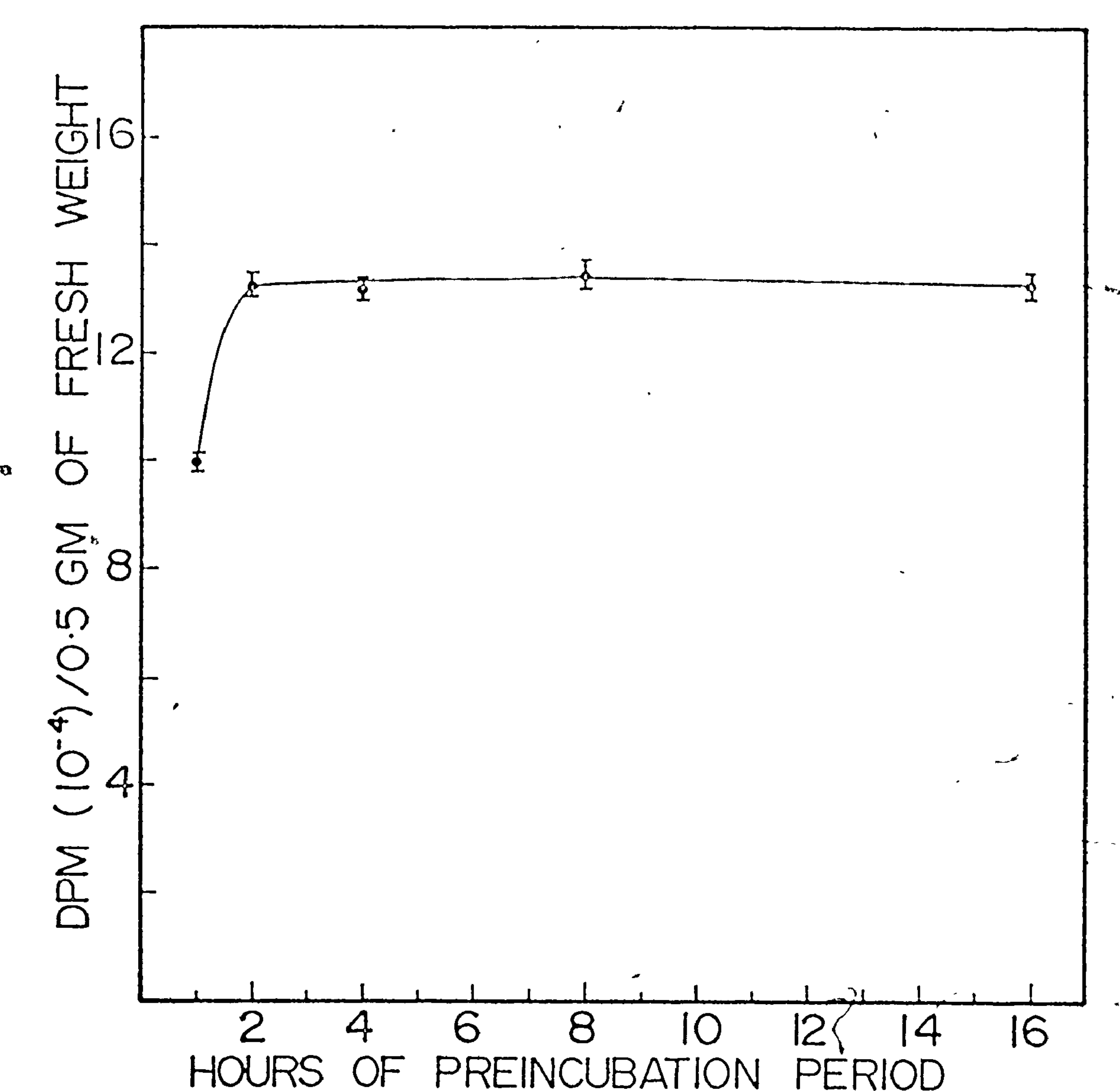


Figure 27

Figure 28. Uptake of [8-^{14}C] i^{6}Ado in KX and 0-1 tissues

Samples (0.5 gm) of KX and 0-1 tissues were suspended in their own basal mediums, minus the agar and the cytokinin (in the case of KX tissue), and were preincubated for 4 hours at 27°C. About 8.0 x 10^{4} dpm of [^{14}C] i^{6}Ado (specific activity 23.5 mCi/mmol) was added and the incubation was continued for different periods of time as shown in the figure. Each sample was then filtered through a Millipore filter and the tissue washed thoroughly with 50 μM i^{6}Ado solution. The radioactivity in both tissues, after alkali ~~and acid~~ hydrolysis (above the horizontal line (A)) and the combined incubation medium plus the washing solution (below the horizontal line (B)) was determined. Duplicate samples were run for each period for both tissues. The vertical bars represent the standard deviation. The experiment was repeated twice with a similar pattern of results being obtained. ▨, KX tissue; ☐, 0-1 tissue.

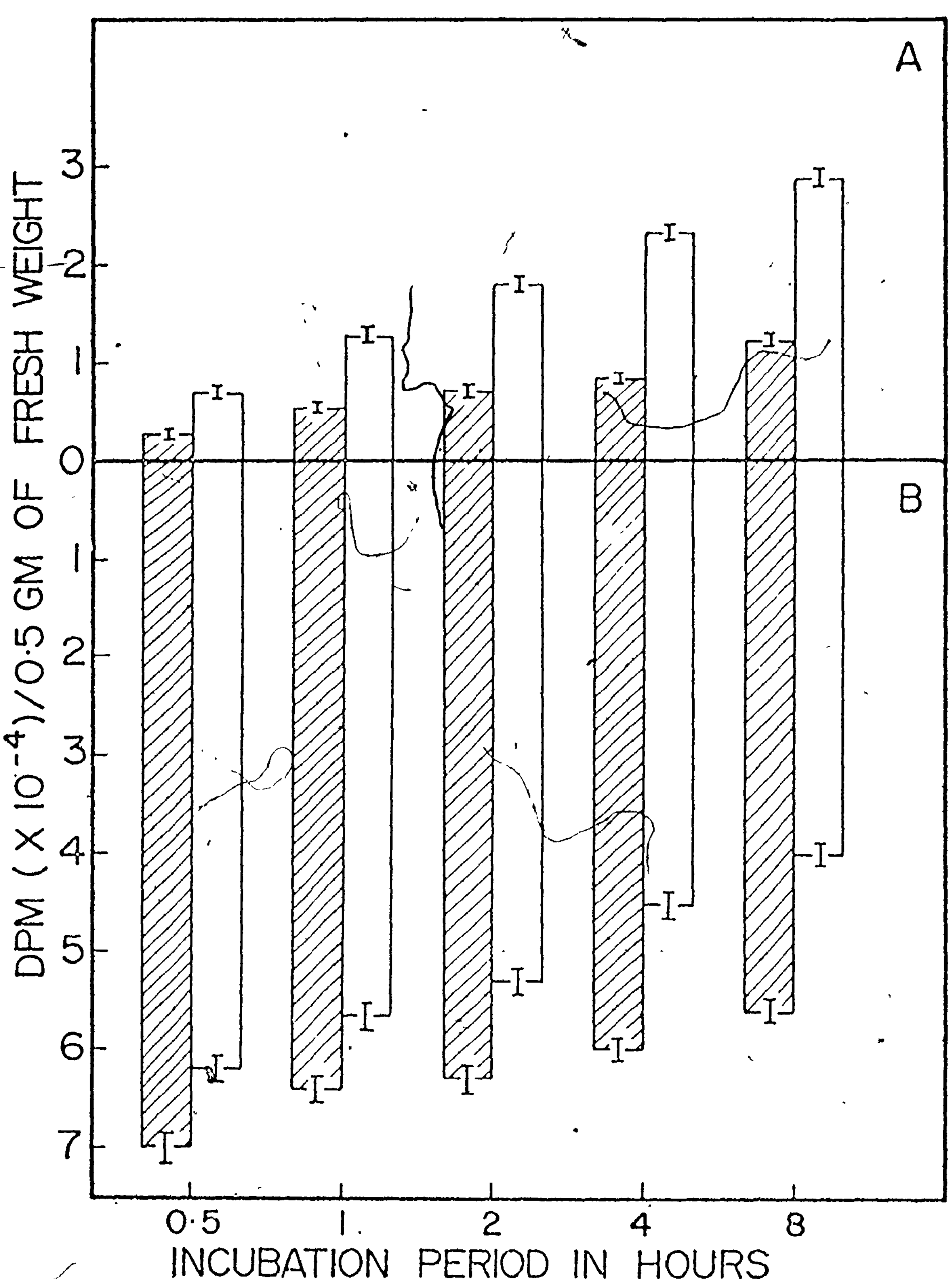


Figure 28

rate of uptake of $[^{14}C]i^6Ado$ by O-1 tissue is much higher than that of KX tissue. For both tissues, however, the amount taken up was still increasing after 8 hours of incubation. Another observation was that in both cases after 8 hours of incubation, much of the radioactivity remained in the incubation medium. This could be due to a peculiar property of the plant hormone or the uptake of $[^{14}C]i^6Ado$ might be different from the uptake of unmodified nucleoside by the same tissue.

This assumption was tested by performing uptake studies of $[^{14}C]$-i^6Ado, $[^{14}C]Ado$ and $[^{14}C]Ade$ at equal concentration by identical amounts of KX tissue (Table 4 and Figure 29). The rate of uptake of $[^{14}C]i^6Ado$ was three times slower than that of $[^{14}C]Ado$ and $[^{14}C]Ade$. Therefore, the uptake of i^6Ado may be regulated in a different manner than the other two compounds.

Further, the influence of ribosyl kinetin on the uptake of $[^{14}C]$-i^6Ado by KX tissue was studied in order to understand the relationship between the two compounds and the nature of the uptake of i^6Ado (Table 5). It was found that the increasing level of the synthetic cytokinin gradually reduced the uptake of the natural cytokinin in KX tissue.

(iii) <u>Studies on the accumulation and disappearance of metabolites</u>

In order to make a comparative study of i^6Ado metabolism in both tissues, the ethanol soluble metabolites were separated and the amount of each determined as described in the Methods section. The quantitative measurements of these compounds are shown in Figures 30, 31, 32 and 33. The first two figures indicate the accumulation and the following

TABLE 4

Comparative studies on the uptake of [^{14}C] i^6Ado, [^{14}C] Ado and [^{14}C] Ade by KX tissue

Compound	Dpm added (Spec. Act.)	Concentration in the medium	Rate of uptake	% Recovery
[^{14}C] i^6Ado	3.0×10^5 (23.5)	5.8×10^{-6}M	1.81	87
[^{14}C] Ado	3.57×10^5 (23.5)	6.9×10^{-6}M	5.24	89
[^{14}C] Ade	9.79×10^5 (62.0)	7.2×10^{-6}M	6.08	90

A sample of 0.5 g of KX tissue was preincubated for four hours at 27°C in 1.0 ml of its basal medium, except the agar and plant hormones. The above mentioned amount of label compounds were added and incubation was continued for another two hours. These manipulations were done under sterile conditions. The radioactivity taken up by the tissue was determined after hdrolyzing the tissue in 2.0M KOH and then in 2.0M HCl, as described in the Experimental section. Duplicate samples were run for each compound and the means were taken.

The rate of uptake is expressed in nmol/h/g of fresh weight of the tissue.

Figure 29. Comparative studies on the uptake of [^{14}C] i^{6}Ado, [^{14}C] Ado and [^{14}C] Ade by KX tissue

The results of the previous experiment are presented in a different way. The description is the same as that shown at the bottom of Table 4.

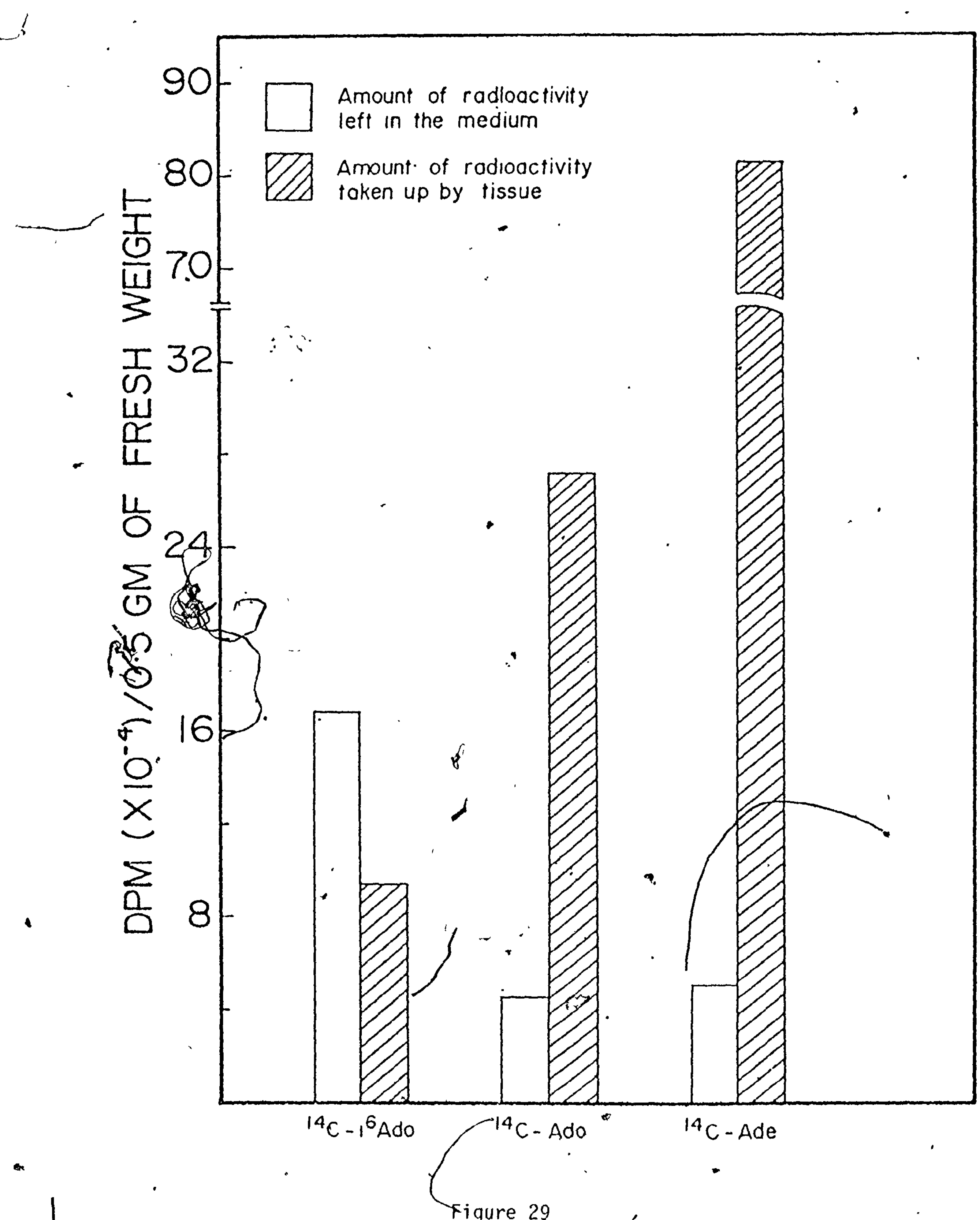


Figure 29

TABLE 5

Influence of ribosyl kinetin on the uptake of $[^{14}C]$ i^6 Ado in KX tissue

Sample	Concentration of ribosyl kinetin	$[^{14}C]$ i^6Ado taken up by the tissue dpm/g of fresh wt.	% of radioactivity added
Control	-	220,378 ± 2678	39.92
Expt. No. 1	3×10^{-7}M	143,453 ± 2204	25.98
Expt. No. 2	3×10^{-6}M	107,446 ± 1832	19.46
Expt. No. 3	3×10^{-5}M	64,885 ± 1486	11.75

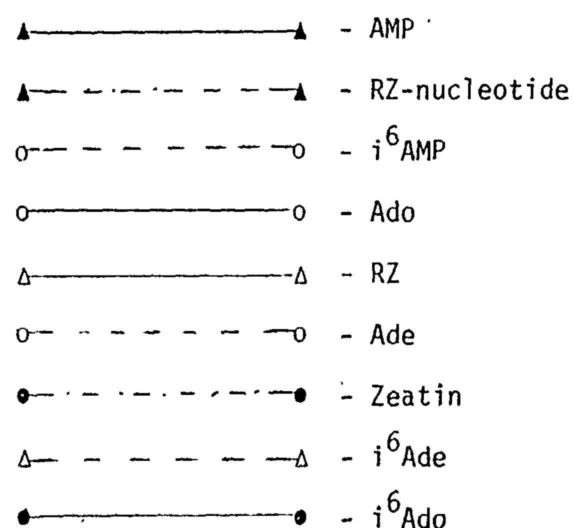
Five g of tissue was preincubated in its own basal medium minus the agar, for four hours at 27°C. The amounts of ribosyl kinetin listed above were included in respective samples. About 5.5 x 10^5 dpm of $[^{14}C]$ i^6Ado was added and incubation was continued for another four hours. These procedures were carried out under sterile conditions. Each sample was washed thoroughly and the total amount of $[^{14}C]$ i^6Ado taken up by the tissue was determined after alkali and acid hydrolysis as described in the Method section. Duplicate samples were run for each ribosyl kinetin concentration. The experiment was repeated and similar results were obtained. The results are expressed in Mean ± S.D.

Figure 30. Accumulation of i^6Ado and its metabolites in KX tissue

Five grams of KX tissue was preincubated in its own basal medium, minus the agar and kinetin, for 4 hours at 27°C. About 1.46×10^6 dpm of [^{14}C] i^6Ado was added and incubation was continued as shown in the figure. These procedures were done under sterile conditions.

At the end of each incubation period, the tissue was washed and extracted with 80% ethanol as described in the Method section. The ethanol-soluble metabolites were separated first by an LH-20 column and followed by paper chromatography. After scanning the paper in the Actigraph III, the radioactive compounds were eluted and counted in a Nuclear Chicago liquid scintillation counter.

Duplicate samples were run for each determination and the means were taken for presentation. The experiment was repeated with similar results.



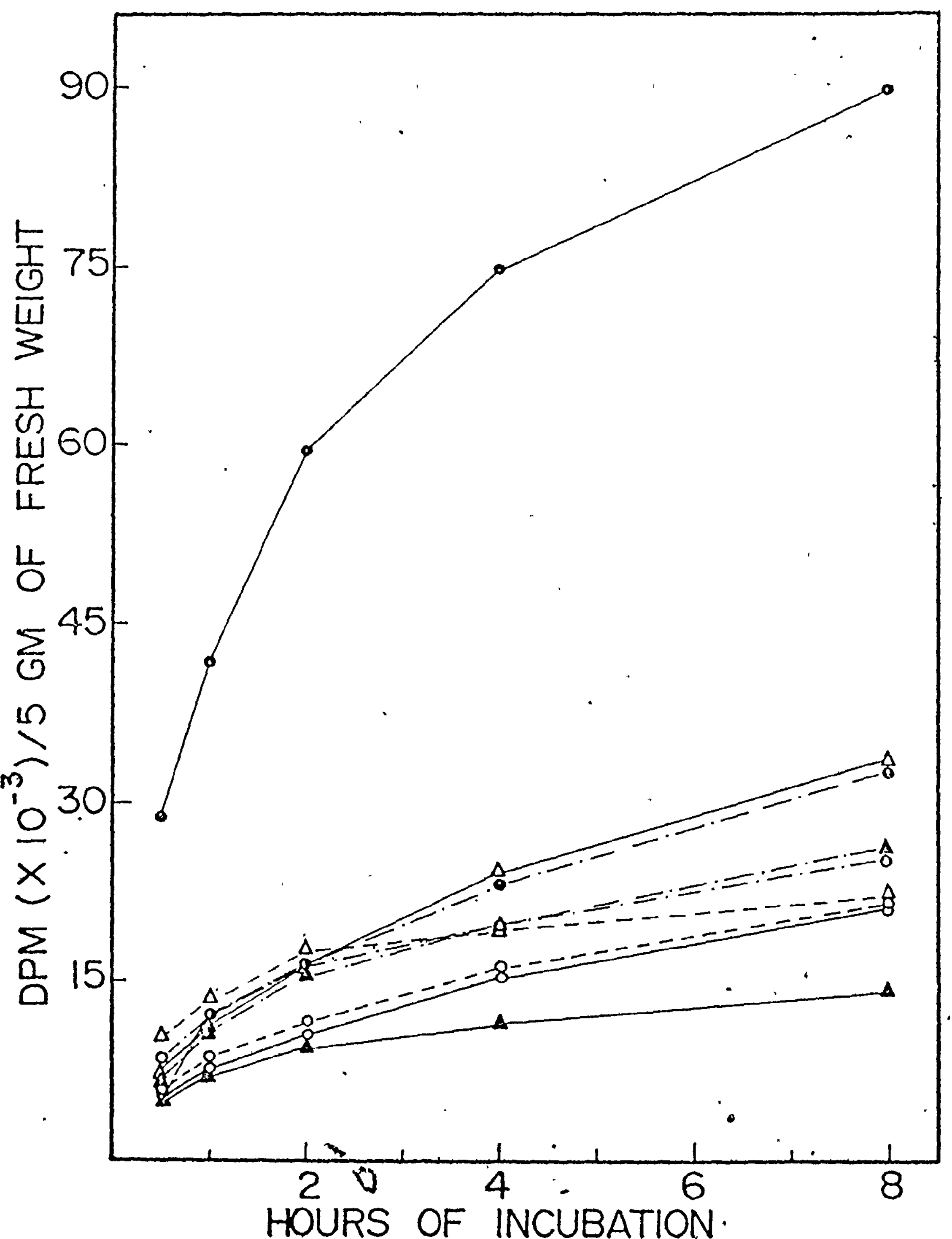
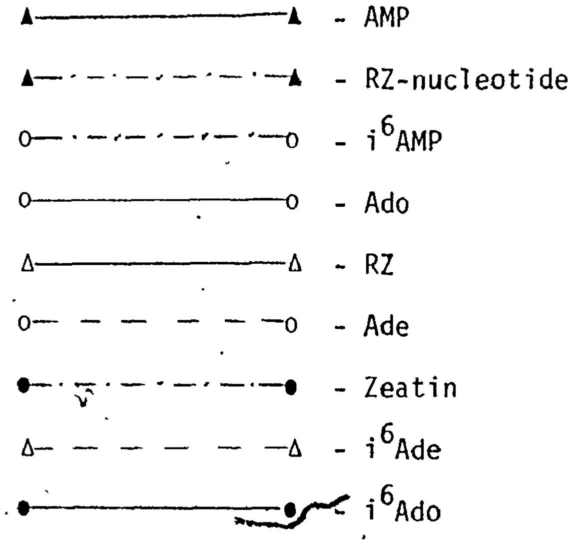


Figure 30

Figure 31. Accumulation of i^6Ado and its metabolites in O-1 tissue

Five grams of O-1 tissue was preincubated in its own basal medium, minus the agar, for 4 hours at 27°C. About 1.4×10^6 dpm of i^6Ado was added and incubation was continued as shown in the figure. These manipulations were done under sterile conditions.

The extraction and the determination of metabolites for each sample were the same as that of Figure 30. Duplicate samples were run for each determination and the means were taken for presentation. The experiment was repeated and similar results were obtained.



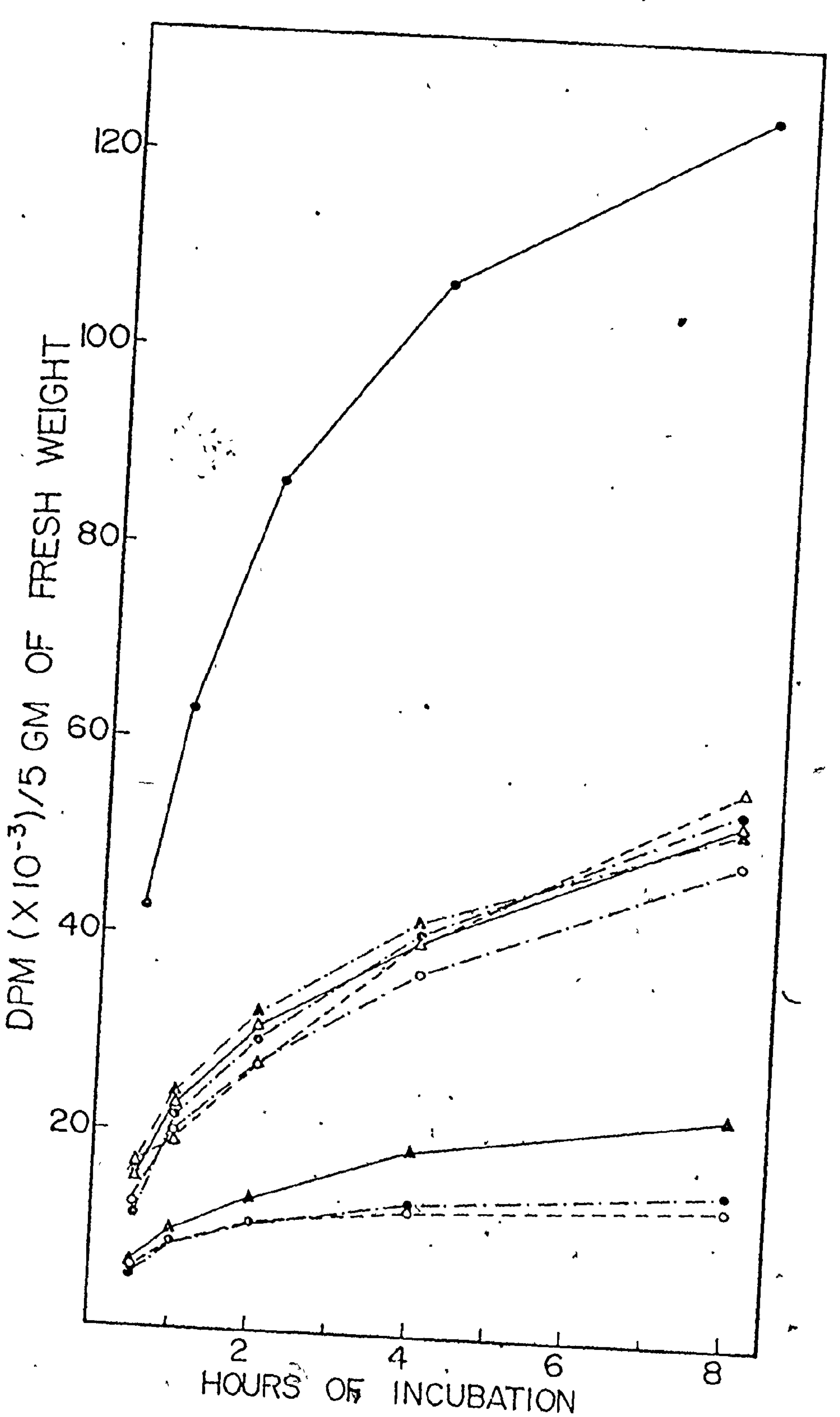


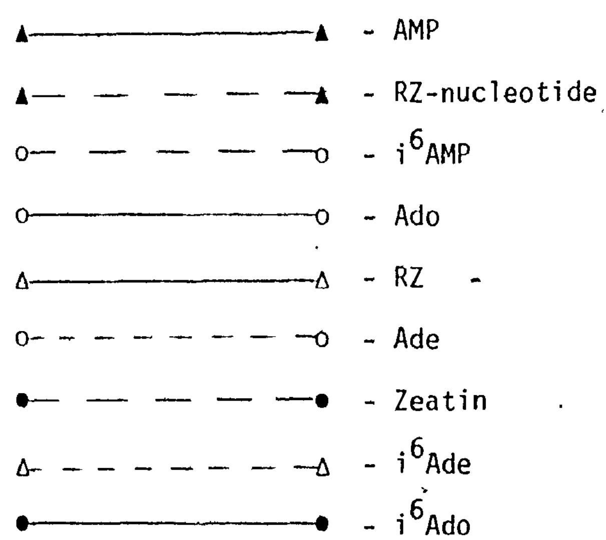
Figure 31

Figure 32. Disappearance of i^6Ado and its metabolites in KX tissue

Five grams of KX tissue was preincubated in its own basal medium, minus agar and ribosyl kinetin, for 4 hours at 27°C. About 1.9 x 10^6 dpm of [^{14}C] i^6Ado was added and incubation was continued for another 4 hours. The concentration of i^6Ado in the 10 ml of medium was 3.7 x 10^{-6}M. These procedures were carried out under sterile conditions.

The tissue was washed thoroughly with phosphate buffer (0.05 M, pH 7.0) containing 50 μM of unlabelled i^6Ado. It was immediately transferred into fresh incubation medium, which also contained unlabelled i^6Ado at a concentration of 8.0 x 10^{-6}M. One sample of tissue was extracted with 80% ethanol and was considered as a zero hour sample.

At the end of the second incubation period, the tissue was extracted with 80% ethanol. All samples of the ethanol soluble metabolites were fractionated and determined as shown in the Method section. Duplicate samples were run for determination and the means were taken for presentation. The experiment was repeated and similar results were obtained.



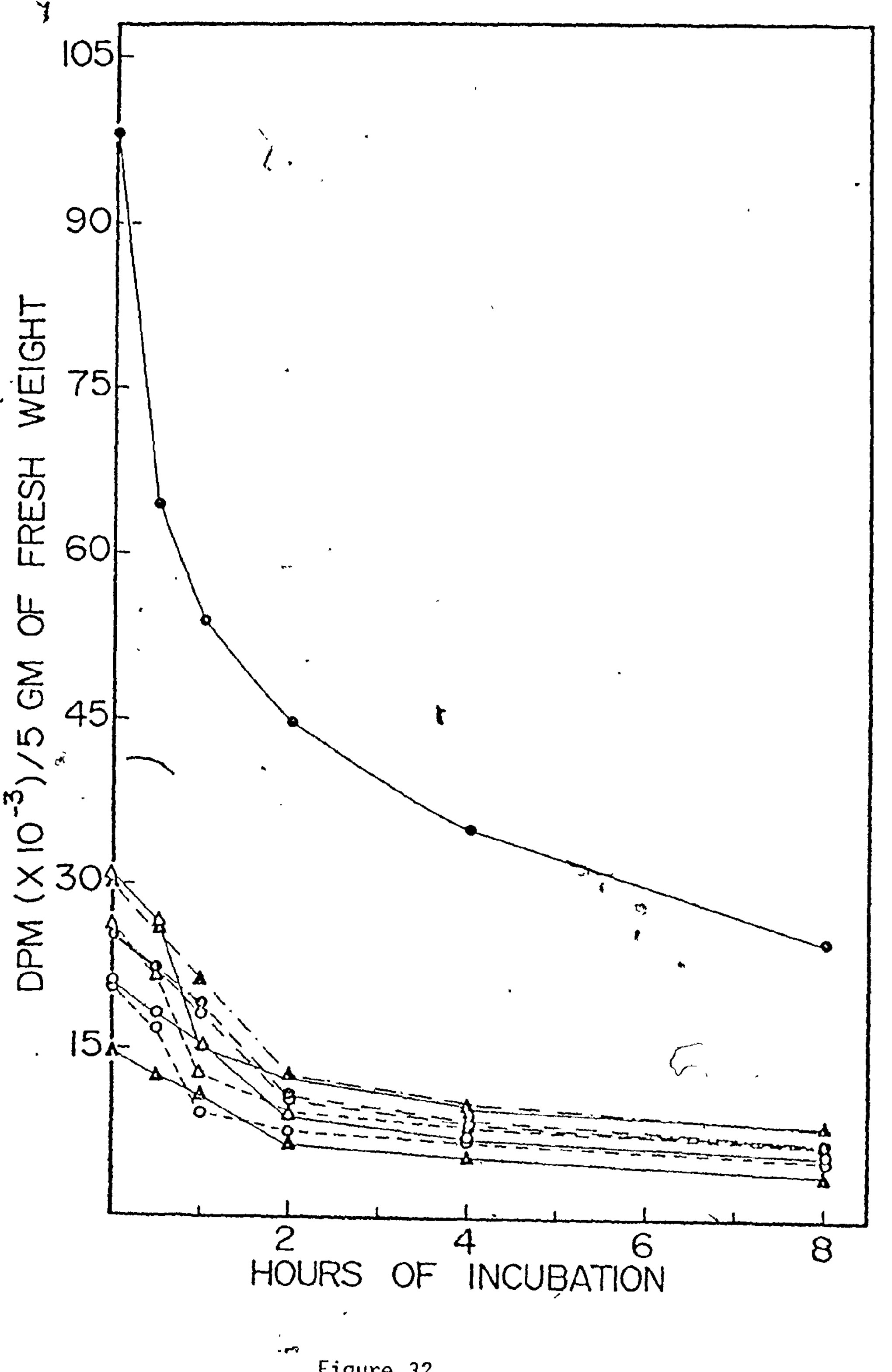
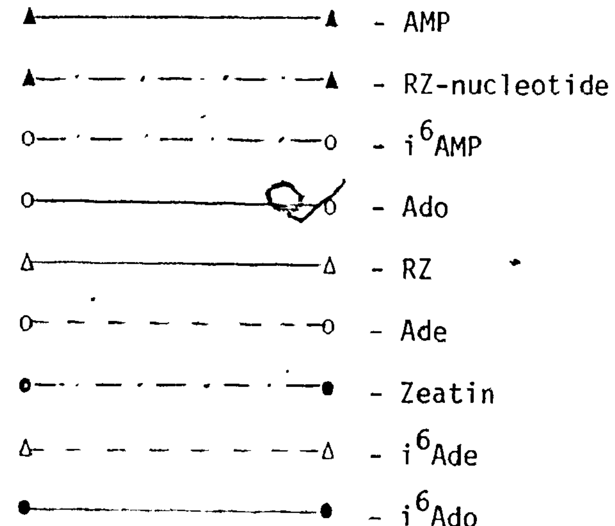


Figure 32

Figure 33. Disappearance of i^6Ado and its metabolites in 0-1 tissue

Five grams of 0-1 tissue was preincubated in its own basal medium, minus the agar, for 4 hours at 27°C. About 2.04×10^6 dpm of [^{14}C] i^6Ado was added and the incubation was continued for another 4 hours. The concentration of i^6Ado in the 10 ml of incubation medium was 3.9×10^{-6}M. These procedures were done under sterile conditions.

From here on the description was the same as that of Figure 32.



160
140
120
100
80
60
40
20

DPM (X10^{-3})/5 GM OF FRESH WEIGHT

2 4 6 8

HOURS OF INCUBATION

Figure 33

two figures show the disappearance of i^6Ado and its metabolites in KX and O-1 tissues, respectively. These figures by themselves do not demonstrate any significant features of i^6Ado metabolism in these two tissues. When the respective compounds, however, were compared individually for both accumulation and disappearance, the quantitative difference in metabolism could be clearly seem (Figures 34 to 39).

In all cases, the accumulation of metabolites in O-1 tissue was much faster than that of KX tissue. This finding indicates that the intracellular pool sizes of the metabolites is higher in the autonomous tissue than in the normal tissue, because not only a higher level of the compounds accumulated after 8 hours of incubation, but a higher level was still detectable after 8 hours of chase. However, the initial rate of disappearance in O-1 tissue appeared to be similar to that of KX tissue.

3. INTERHORMONAL EFFECT STUDIES

Having shown that there are differences in i^6Ado metabolism, I studied the interaction of the cytokinin and other plant hormones in KX and O-1 tissues. First, the influence of auxin on the uptake of [^{14}C]-i^6Ado by both tissues was studied (Table 6). It was found that the NAA concentration at 5 x 10^{-7}M increased the uptake of [^{14}C]i^6Ado in KX tissue, whereas 5 x 10^{-5}M concentration of NAA did not change the uptake of the compound. The NAA concentration at 5 x 10^{-6}M was considered normal, because

Figure 34. Comparative studies of i^6AMP in KX and O-1 tissues.

Rates of accumulation and disappearance of i^6AMP in KX and O-1 tissues from Figures 30, 31, 32 and 33 were selected and presented here for comparison. Vertical bars represent the standard deviation of the means.

A : Rate of accumulation

B : Rate of disappearance

●————● - O-1 tissue

o – – – – o - KX tissue

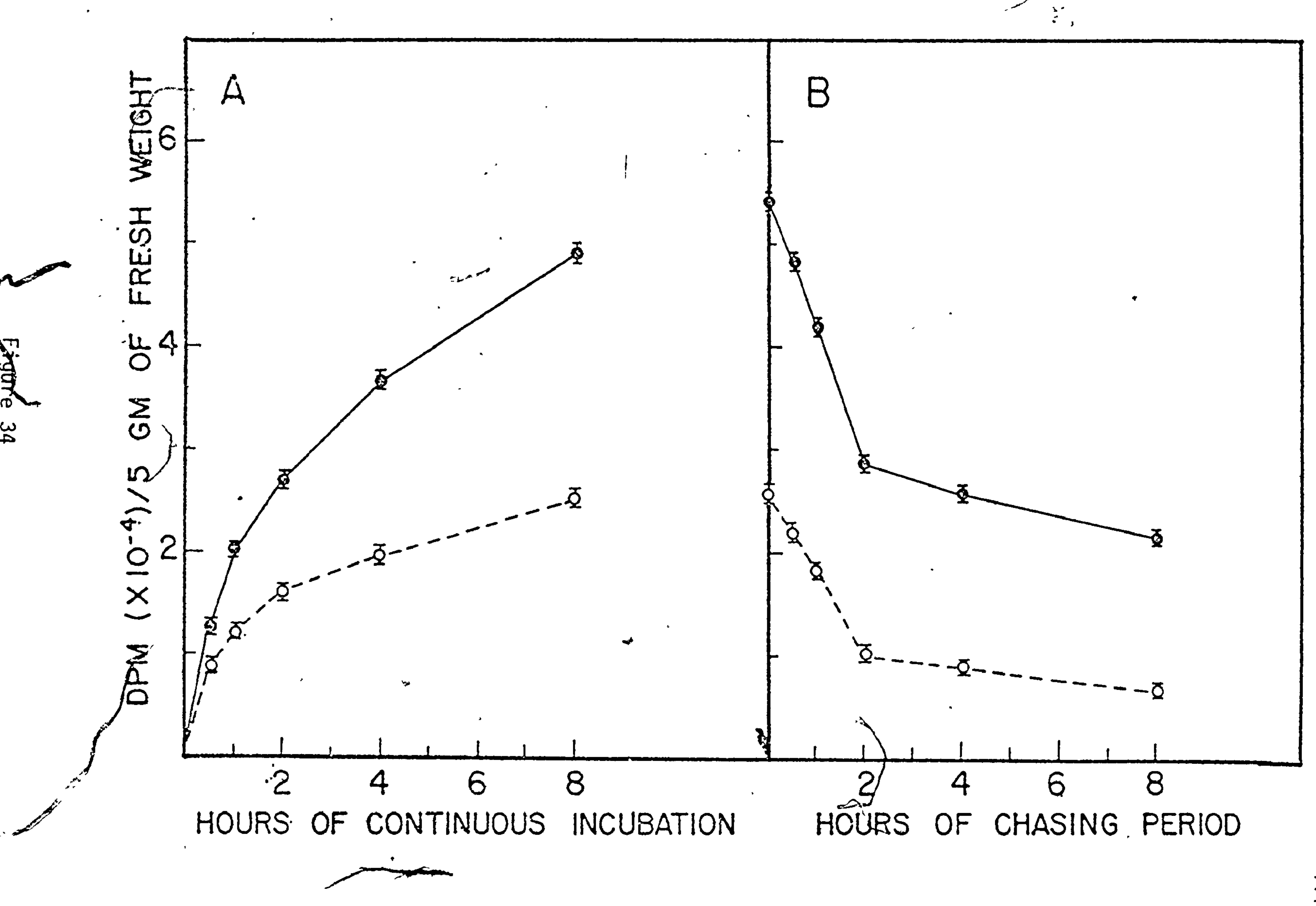


Figure 34

Figure 35. Comparative studies of RZ-nucleotide in KX and O-1 tissues.

Rates of accumulation and disappearance of RZ-nucleotide in KX and O-1 tissues from Figures 30, 31, 32 and 33 were selected and presented here for the comparison. Vertical bars represent the standard deviation of the means.

A : Rate of accumulation

B : Rate of disappearance

●——● - O-1 tissue

o – – – o - KX tissue

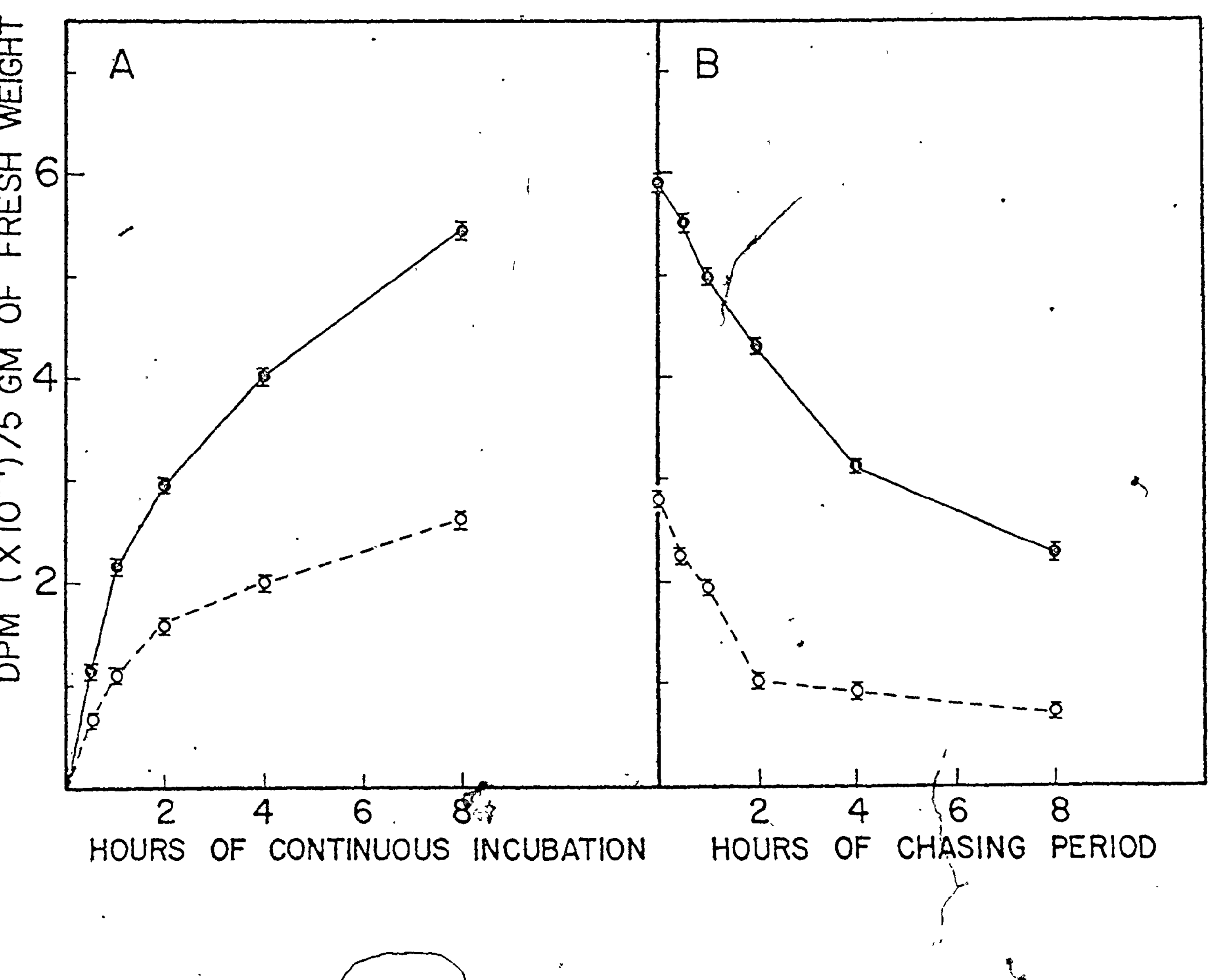


Figure 35

Figure 36. Comparative studies of RZ in KX and O-1 tissues.

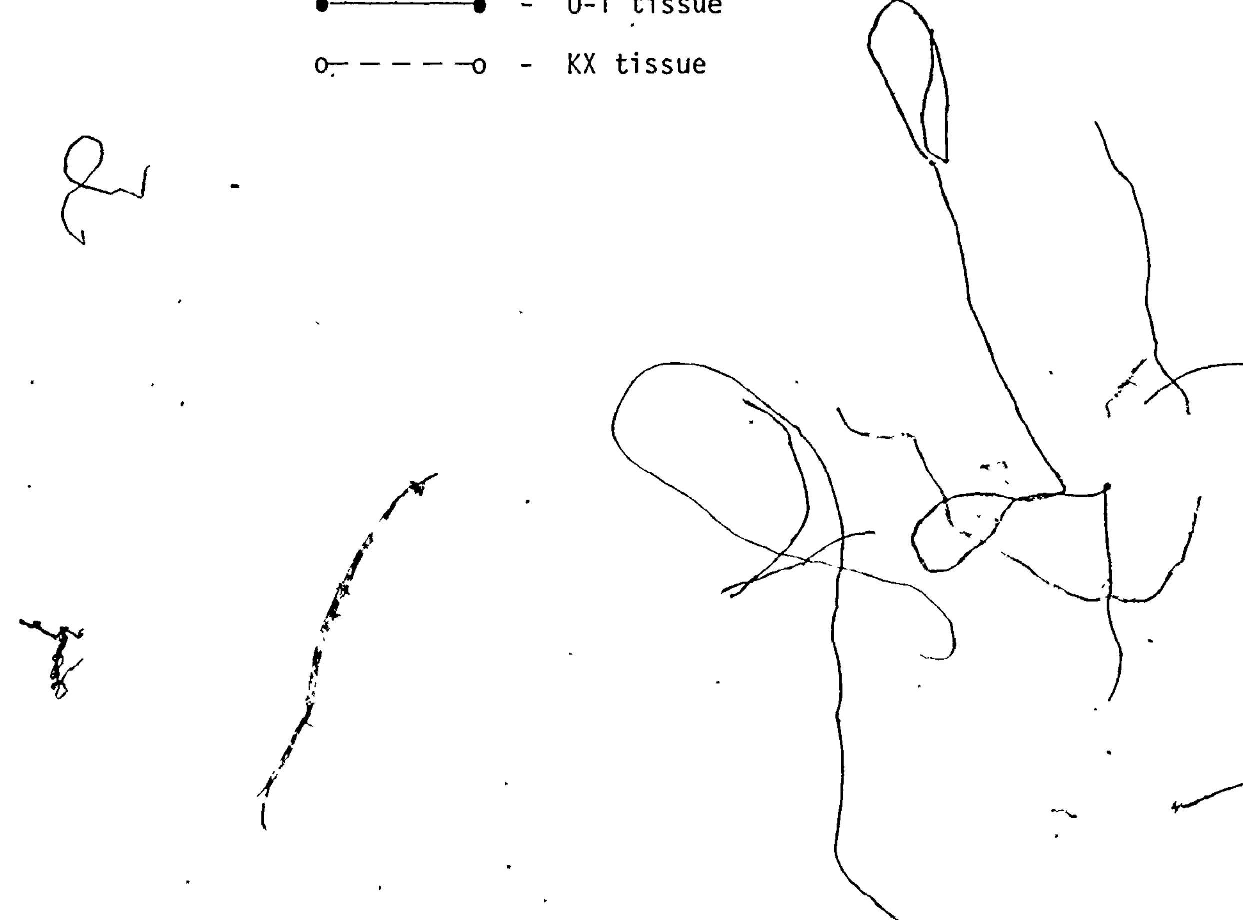
Rates of accumulation and disappearance of RZ in KX and O-1 tissues from Figures 30, 31, 32 and 33 were selected and presented here for comparison. Vertical bars represent the standard deviation of the means.

A : Rate of accumulation

B : Rate of disappearance

●———● - O-1 tissue

o- - - - -o - KX tissue



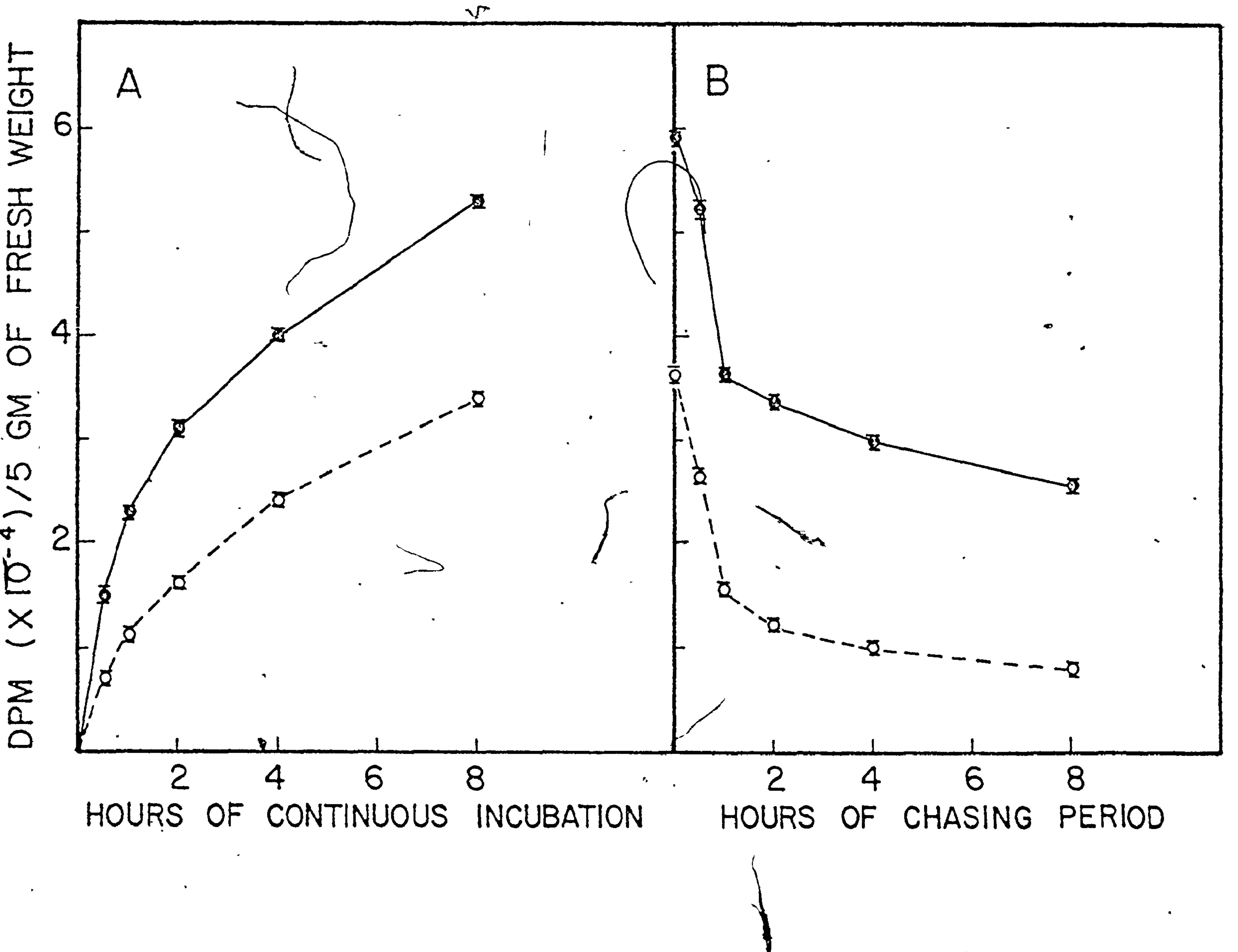


Figure 36

Figure 37. Comparative studies of zeatin in KX and 0-1 tissues.

Rates of accumulation and disappearance of zeatin in KX and 0-1 tissues from Figures 30, 31, 32 and 33 were selected and presented here for comparison. Vertical bars represent the standard deviation of the means.

A : Rate of accumulation

B : Rate of disappearance

●———● - 0-1 tissue

o– – – –o - KX tissue

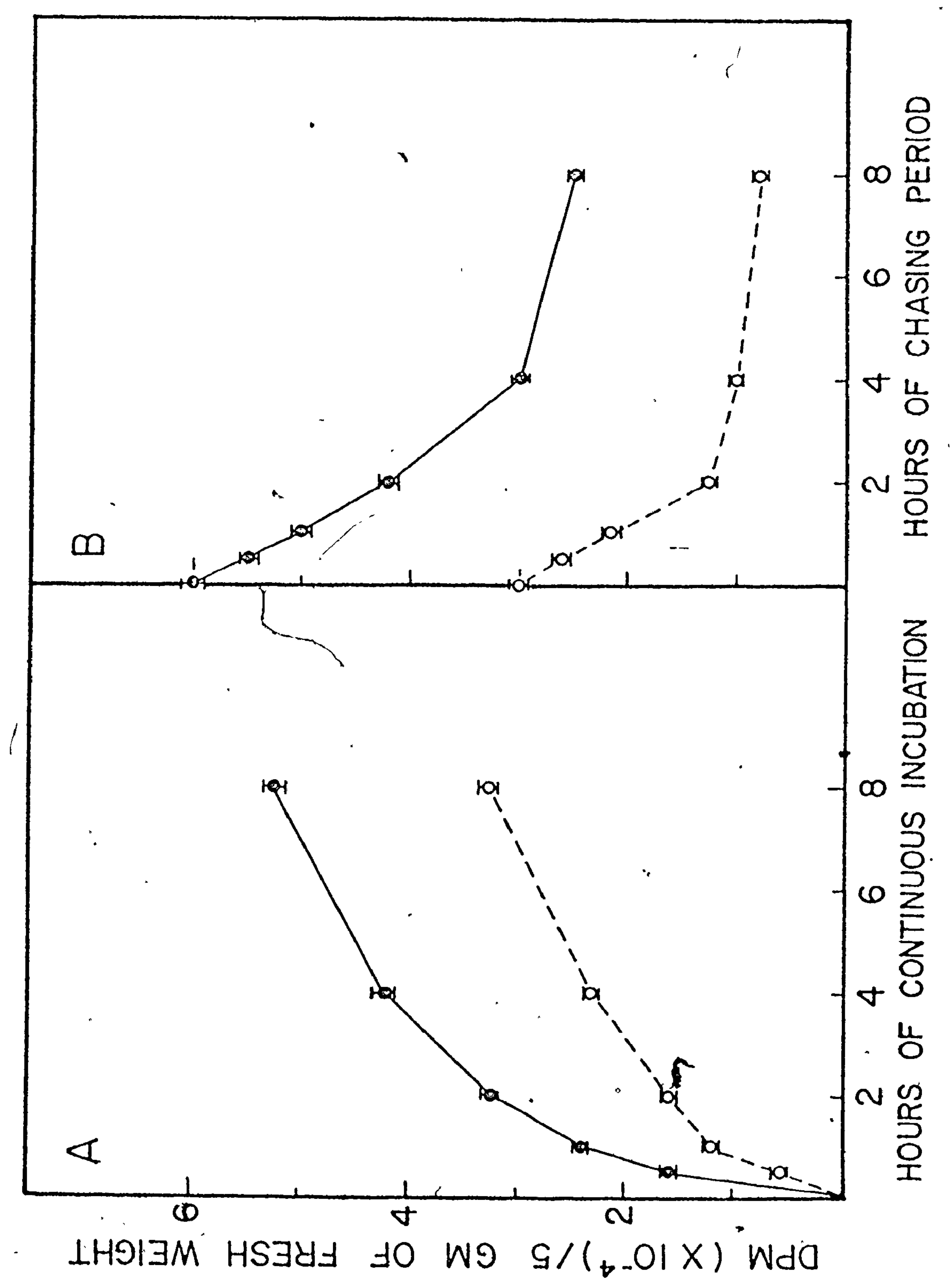


Figure 37

Figure 38. Comparative studies of i^6Ado in KX and O-1 tissues.

Rates of accumulation and disappearance of i^6Ado in KX and O-1 tissues from Figures 30, 31, 32 and 33 were selected and presented here for comparison. Vertical bars represent the standard deviation of the means.

A : Rate of accumulation

B : Rate of disappearance

●————● - O-1 tissue

o– – – – –o - KX tissue

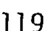


Figure 38

Figure 39. Comparative studies of i^6Ade in KX and O-1 tissues

Rates of accumulation and disappearance of i^6Ade in KX and O-1 tissues from Figures 30, 31, 32 and 33 were selected and presented here for comparison. Vertical bars represent the standard deviation of the means.

A : Rate of accumulation

B : Rate of disappearance

●———● - O-1 tissue

o- - - - -o - KX tissue

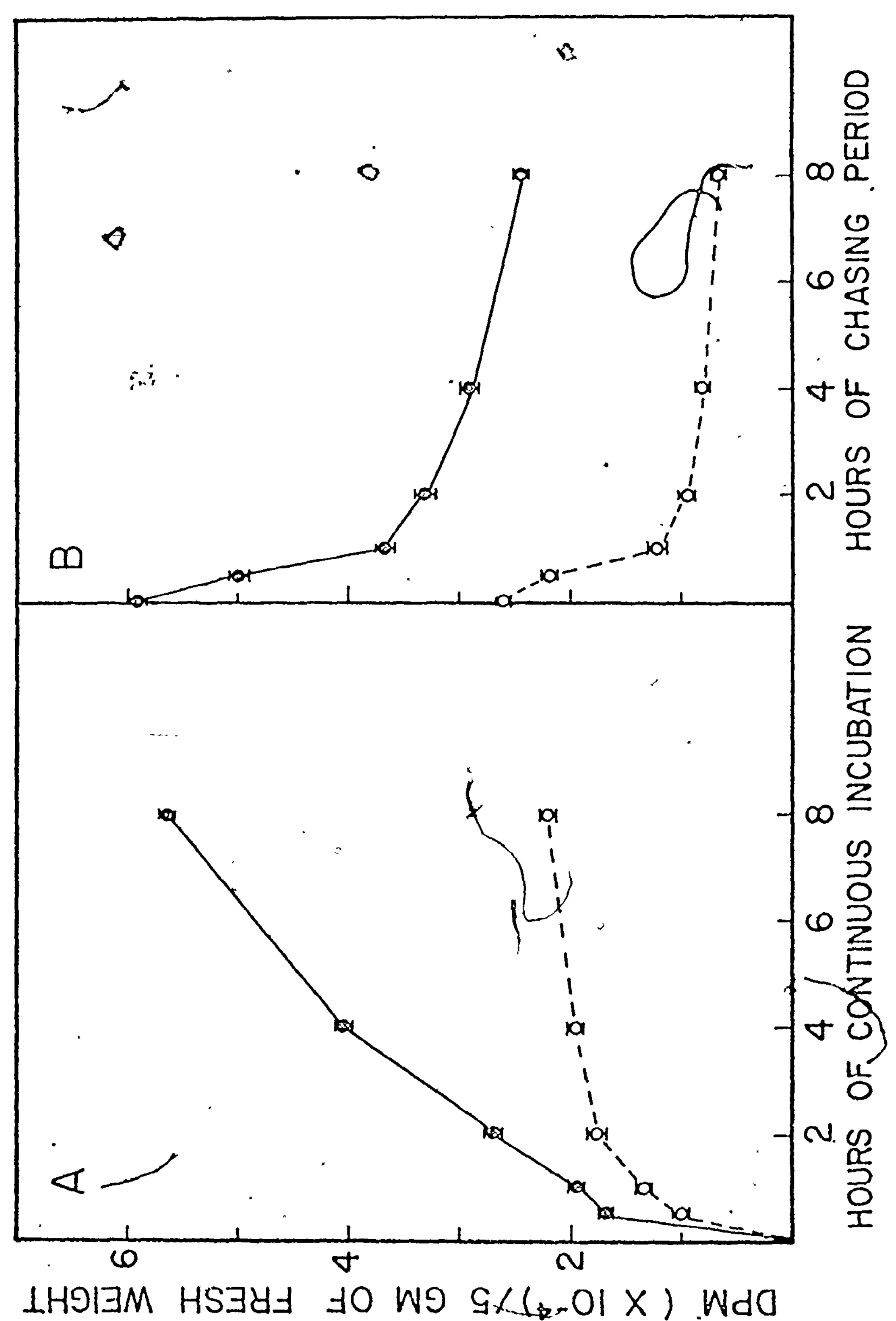


Figure 39

TABLE 6

Influence of auxin on the uptake of [^{14}C] i^6Ado by the tobacco tissues

Tissue	NAA concentration in incubation medium	[^{14}C] i^6Ado taken up by the tissues dpm/g fresh wt.	% of radioactivity added
KX	5×10^{-7}M	201,128 ± 2626	36.47
	5×10^{-6}M	90,198 ± 1758	16.35
	5×10^{-5}M	92,892 ± 1786	16.84
0 - 1	5×10^{-7}M	231,592 ± 2806	41.99
	5×10^{-6}M	239,220 ± 2864	43.38
	5×10^{-5}M	222,494 ± 2760	40.34

Five g of KX and 0 - 1 tissues were preincubated in their own basal mediums minus agar for four hours at 27°C. A concentration of NAA listed above was included in the respective samples. About 5.5×10^5 dpm of [^{14}C] i^6Ado was added to each sample and incubation was continued for another four hours. These manipulations were done under sterile conditions. The tissues were washed thoroughly and the total amount of radioactivity taken up by the tissues was determined after alkali and acid hydrolysis as mentioned above. Duplicate samples were run for each NAA concentration for both tissues. The experiment was repeated and similar results were obtained. The results are expressed in Mean ± S.D.

it was the same concentration that had been used in the basal medium for the KX tissue. In O-1 tissue, however, the different concentrations of NAA had no effect on the uptake of the cytokinin.

i) Influence of NAA on i^6Ado metabolism

It has been reported in the literature that there is a correlation between the uptake and the metabolism of a compound in plant cells. Because certain concentrations of NAA could increase the uptake of [^{14}C]i^6Ado in the KX tissue, the influence of NAA on the metabolism of i^6Ado was studied in both KX and O-1 tissues. Table 7 shows the effect of NAA on the i^6Ado metabolism in KX tissue.

The 10^{-7}M concentration of NAA increased the intracellular level of these compounds, compared with that obtained with 10^{-6}M concentration of NAA. The NAA concentration of 10^{-5}M, however, did not change the level of these compounds. Because the starting compound was [^{14}C]i^6Ado, which was fed to the tissue, the ratios of these metabolites with respect to the i^6Ado pool was recalculated based on the data in Table 7. The results are shown in Table 8. This method of presentation shows more clearly the change in the level of the compounds, AMP, RZ-nucleotide, i^6AMP, Zeatin and i^6Ade at the lowest concentration of NAA.

As might be expected from the data in Table 6, it was found that the intracellular level of metabolites in O-1 tissue did not change appreciably due to the influence of NAA at different concentrations as shown in Tables 9 and 10. Therefore, it appears that auxin has little effect on the i^6Ado metabolism in O-1 tissue, while it shows significant influence in KX tissue.

TABLE 7

Five g samples of KX tissue in its own basal medium, minus the agar and plant hormones, was preincubated for four hours at 27°C. The NAA concentration at 10^{-5}, 10^{-6} and 10^{-7}M were included in the respective incubation medium. About 1.71×10^{6} dpm of [^{14}C] i^{6}Ado was added and incubation was continued for another eight hours. These manipulations were done under sterile conditions. At the end of the incubation period, the tissue was washed and extracted with 80% ethanol. The ethanol soluble metabolites were separated by LH-20 column and paper chromatography and were determined by scintillation counting. Duplicate samples were run for each NAA concentration and the means were taken. The results are expressed in Mean ± S.D. The experiment was repeated and similar results were obtained.

TABLE 7

Influence of NAA on i^6Ado metabolism in KX tissue

Compounds	Concentration of NAA		
	10^{-7}M	10^{-6}M	10^{-5}M
AMP	32,690 ± 432	12,042 ± 262	11,358 ± 255
$Unknown_1$	13,510 ± 278	7,773 ± 210	7,362 ± 205
RZ - nucleotides	60,678 ± 589	21,947 ± 354	20,825 ± 345
i^6AMP	56,936 ± 570	21,337 ± 349	19,072 ± 330
$Unknown_2$	13,752 ± 284	7,082 ± 201	8,078 ± 215
Ado	32,328 ± 430	15,286 ± 296	17,984 ± 321
RZ	46,322 ± 514	25,874 ± 384	28,805 ± 406
$Unknown_3$	14,118 ± 284	8,001 ± 214	7,713 ± 210
Ade	31,846 ± 426	18,746 ± 327	17,670 ± 311
Zeatin	50,432 ± 537	27,890 ± 399	25,102 ± 379
i^6Ade	44,874 ± 716	18,442 ± 459	16,688 ± 437
i^6Ado	120,628 ± 1174	76,204 ± 933	70,118 ± 895

TABLE 8

Changes in the relative ratios of metabolites with respect to the i^6Ado pool due to different concentration of NAA in KX tissue

Compounds	Concentration of NAA		
	10^{-7}M	10^{-6}M	10^{-5}M
AMP	0.271	0.158	0.162
$Unknown_1$	0.112	0.102	0.105
RZ - nucleotide	0.503	0.288	0.297
i^6AMP	0.472	0.280	0.272
$Unknown_2$	0.114	0.106	0.101
Ado	0.268	0.236	0.218
RZ	0.384	0.378	0.369
$Unknown_3$	0.117	0.105	0.110
Ade	0.252	0.246	0.252
Zeatin	0.418	0.366	0.358
i^6Ade	0.372	0.242	0.238
i^6Ado (Reference)	1.000	1.000	1.000

The results of Table 7 presented in a different form.

TABLE 9

Influence of NAA on i^6Ado metabolism in 0 - 1 tissue

Compounds	Concentration of NAA		
	10^{-7}M	10^{-6}M	10^{-5}M
AMP	22,403 ± 358	20,476 ± 341	19,813 ± 336
$Unknown_1$	16,947 ± 311	15,358 ± 296	16,512 ± 307
RZ - nucleotide	50,842 ± 529	47,164 ± 519	47,710 ± 522
i^6AMP	46,663 ± 517	43,134 ± 496	43,497 ± 498
$Unknown_2$	11,260 ± 254	8,932 ± 452	11,728 ± 259
Ado	13,813 ± 281	12,635 ± 269	12,411 ± 266
RZ	50,378 ± 536	46,946 ± 518	48,052 ± 524
$Unknown_3$	12,792 ± 270	10,348 ± 243	11,842 ± 260
Ade	13,223 ± 275	13,071 ± 273	14,461 ± 288
Zeatin	49,681 ± 533	45,312 ± 509	45,888 ± 512
i^6Ade	51,190 ± 765	47,055 ± 733	47,596 ± 737
i^6Ado	116,078 ± 1152	108,924 ± 1116	113,866 ± 1140

Experimental procedure was identical to that described in Table 7.

TABLE 10

Changes in the relative ratios of metabolites with respect to i^6Ado pool due to different concentration of NAA on 0 - 1 tissue

Compounds	Concentration of NAA		
	10^{-7}M	10^{-6}M	10^{-5}M
AMP	0.193	0.188	0.174
$Unknown_1$	0.146	0.141	0.145
RZ - nucleotide	0.438	0.433	0.419
i^6AMP	0.402	0.396	0.382
$Unknown_2$	0.097	0.082	0.103
Ado	0.119	0.116	0.109
RZ	0.432	0.431	0.422
$Unknown_3$	0.102	0.095	0.104
Ade	0.114	0.120	0.127
Zeatin	0.423	0.416	0.403
i^6Ade	0.441	0.432	0.418
i^6Ado (Reference)	1.000	1.000	1.000

The results of Table 9 presented in a different form.

(ii) Influence of ABA on i^6Ado metabolism

The data in Tables 11 and 13 show the effect of ABA on i^6Ado metabolism in KX and O-1 tissues, respectively. The ratios of these metabolites with respect to i^6Ado pool is also shown in Tables 12 and 14. The metabolism in KX tissue was found to be perturbed by the different concentration of ABA. Levels of some compounds were decreased (e.g. RZ-nucleotide and i^6AMP), while levels of certain compounds were increased (e.g. Ado) due to the increasing level of ABA. ABA, however, had no significant effect on the intracellular levels of these metabolites in O-1 tissue.

TABLE 11

Five g of KX tissue in its own basal medium, minus the agar and the plant hormones, was preincubated for four hours at 27°C. The ABA concentration at 10^{-5}, 10^{-6} and 10^{-7}M were included in the respective samples. About 1.62×10^6 dpm of [^{14}C] i^6Ado was then added to each sample and the incubation was continued for another eight hours. These manipulations were done under sterile conditions. Extraction and determination of ethanol soluble metabolites were conducted as before. The results were expressed in Means ± S.D. The experiment was repeated with similar results being obtained.

TABLE 11

Influence of ABA on i^6Ado metabolism in KX tissue

Compounds	Concentration of ABA		
	10^{-7}M	10^{-6}M	10^{-5}M
AMP	9,821 ± 237	10,163 ± 241	8,220 ± 217
$Unknown_1$	6,567 ± 194	5,945 ± 184	3,539 ± 142
RZ - nucleotides	18,214 ± 323	18,252 ± 323	13,301 ± 276
i^6AMP	17,963 ± 320	17,905 ± 320	12,445 ± 267
$Unknown_2$	7,090 ± 201	9,540 ± 233	8,919 ± 237
Ado	14,114 ± 284	16,869 ± 310	15,984 ± 302
RZ	24,781 ± 376	27,100 ± 393	22,720 ± 360
$Unknown_3$	6,699 ± 196	6,775 ± 197	5,252 ± 173
Ade	15,675 ± 299	16,316 ± 292	13,073 ± 273
Zeatin	23,675 ± 368	24,543 ± 374	19,866 ± 337
i^6Ade	15,610 ± 423	16,177 ± 430	13,130 ± 387
i^6Ado	23,675 ± 368	69,134 ± 889	57,086 ± 808

TABLE 12

Changes in the relative ratios of metabolites with respect to different concentration of ABA in KX tissue

Compounds	Concentration of ABA		
	10^{-7}M	10^{-6}M	10^{-5}M
AMP	0.151	0.147	0.144
$Unknown_1$	0.101	0.086	0.062
RZ - nucleotide	0.280	0.264	0.233
i^6AMP	0.276	0.259	0.218
$Unknown_2$	0.109	0.138	0.172
Ado	0.217	0.244	0.280
RZ	0.381	0.392	0.398
$Unknown_3$	0.103	0.098	0.092
Ade	0.241	0.236	0.229
Zeatin	0.364	0.355	0.348
i^6Ade	0.240	0.234	0.230
i^6Ado (Reference)	1.000	1.000	1.000

The results of Table 11 presented in a different form.

TABLE 13

Influence of ABA on i^6Ado metabolism in 0 - 1 tissue

Compounds	Concentration of ABA		
	10^{-7}M	10^{-6}M	10^{-5}M
AMP	18,904 ± 329	18,215 ± 323	16,517 ± 307
$Unknown_1$	13,998 ± 278	12,626 ± 269	12,685 ± 269
RZ - nucleotide	41,793 ± 488	39,804 ± 477	36,867 ± 459
i^6AMP	38,421 ± 469	35,756 ± 452	34,038 ± 441
$Unknown_2$	10,014 ± 239	10,312 ± 243	9,399 ± 231
Ado	12,058 ± 262	10,698 ± 247	10,403 ± 244
RZ	41,895 ± 489	40,189 ± 479	38,236 ± 467
$Unknown_3$	11,138 ± 253	10,987 ± 250	9,126 ± 228
Ade	12,306 ± 265	10,891 ± 249	11,316 ± 254
Zeatin	39,750 ± 477	38,166 ± 467	35,722 ± 453
i^6Ade	40,567 ± 681	38,937 ± 642	36,594 ± 662
i^6Ado	102,284 ± 1080	96,378 ± 1049	91,256 ± 1021

Experimental procedure was identical to that described in Table 11.

TABLE 14

Changes in the relative ratios of metabolites with respect to i^6Ado pool due to different concentration of ABA on 0 - 1 tissue

Compounds	Concentration of ABA		
	10^{-7}M	10^{-6}M	10^{-5}M
AMP	0.185	0.189	0.181
$Unknown_1$	0.137	0.131	0.139
RZ - nucleotide	0.409	0.413	0.414
i^6AMP	0.376	0.371	0.373
$Unknown_2$	0.098	0.107	0.103
Ado	0.100	0.111	0.114
RZ	0.348	0.417	0.419
$Unknown_3$	0.109	0.114	0.100
Ade	0.120	0.113	0.124
Zeatin	0.389	0.396	0.391
i^6Ade	0.397	0.404	0.401
i^6Ado (Reference)	1.000	1.000	1.000

The results of Table 13 presented in a different form.

IV. DISCUSSION

A number of studies directed at solving the problem of the mechanism of cytokinin action in plant have been undertaken by several research groups. But, they seem to be inadequate because most of the investigations centre around the single concept that an active metabolite of cytokinin would serve as an initiator of a chain of biochemical reactions leading to an observed physiological phenomenon. Considerable effort has been devoted to the search for an active metabolite of cytokinin, but an appropriate compound which explains its diverse biological responses in experimental tissues has not been discovered. For this reason, the problem has been approached differently based on a concept that there is a "resonant inter-relationship within the metabolic network which as a whole is far more important than a single active metabolite in expressing a cytokinin phenomena" (Hall, 1973).

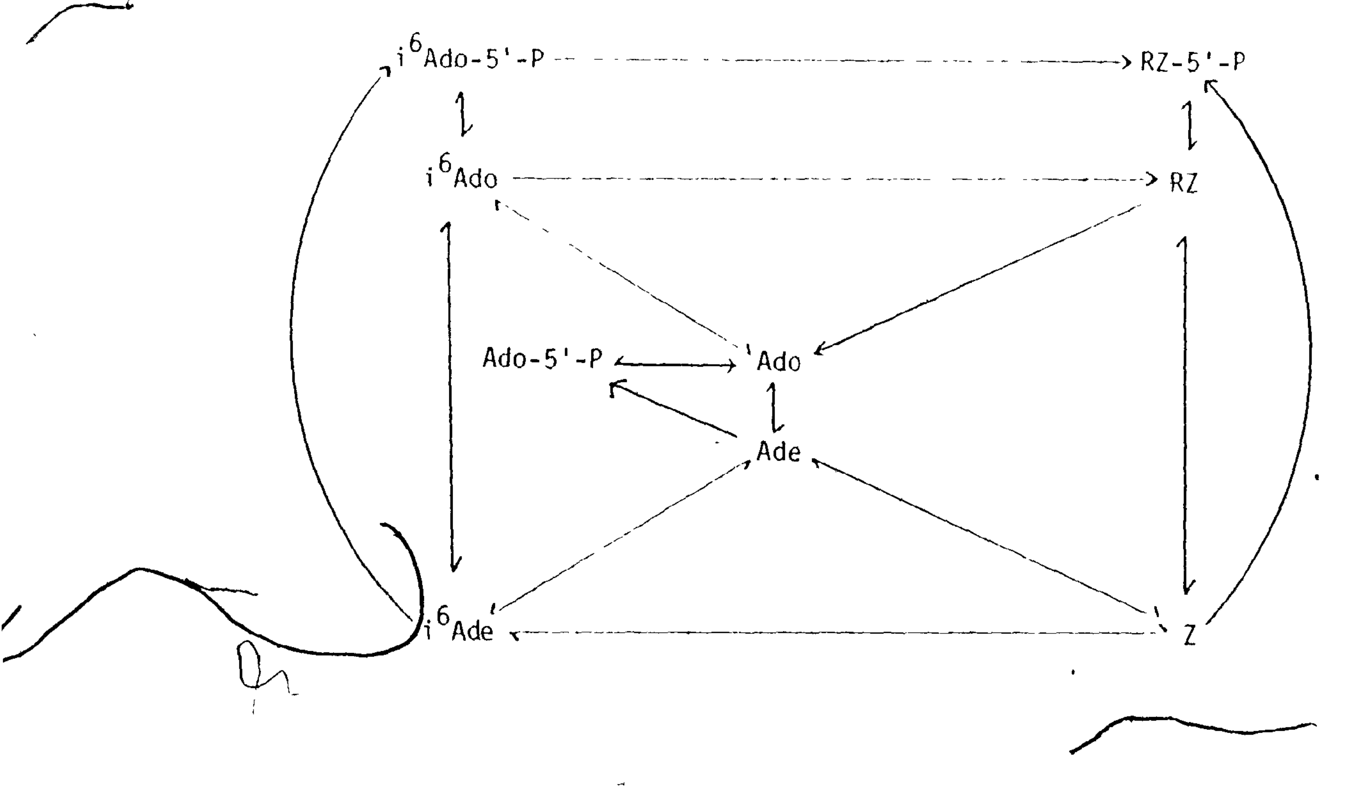
As stated in the Introduction, the first objective of the study was to map out the metabolic pathways of the i^6Ado in both KX and 0-1 tissues. In so doing, it was necessary to study the relationship between the i^6Ado uptake and its metabolism in the tobacco callus, because there is some evidence that the rate of uptake of a compound could influence its metabolism inside a cell. As a prerequisite for the study of this possible relationship, it was necessary to investigate the nature and the rate of i^6Ado uptake by the experimental tissues. This was the second objective. The third and final objective of the study was to explore the nature of the interactions between plant hormones, in this case

cytokinin and auxin, and cytokinin and gibberellic acid.

Qualitative Studies

In order to achieve the first objective, the metabolites of i^6Ado had to be identified in both tissues. The first part of the thesis (Figs. 3 to 23) describes this study, which indicates that the natural cytokinin, i^6Ado, is metabolized extensively in cultured tobacco callus. There are 12 detectable metabolites, nine of which were identified positively as i^6Ado, i^6Ade, RZ, Z, i^6AMP, RZ-nucleotide, AMP, Ado and Ade. Of these compounds, i^6Ado, i^6Ade, RZ and Z were previously shown to exhibit cytokinin activity in this tissue (Skoog and Armstrong, 1970).

Based on the metabolites obtained, the following scheme of metabolism of i^6Ado is proposed.



Assuming the scheme is correct, it can be seen that i^6Ado can be simultaneously converted into four different metabolites, viz. i^6Ade, i^6AMP (or i^6Ado-5'-P), RZ and Ado. The presence of an enzyme that catalyzes the conversion of i^6Ado into Ado or the removal of N^6-side chain from cytokinin compounds, has already been detected in the tobacco callus by Paces *et al.* (1971). This enzyme has been suggested as a controlling factor in maintaining the proper intracellular level of cytokinins in plant tissue (Hall, 1973). An analogous enzyme was isolated from corn kernels and named cytokinin oxidase by Whitty and Hall (1974).

An important point arises here. Although the base, i^6Ade, can be formed directly from i^6Ado by the action of a nucleosidase, the formation of i^6AMP can arise by two different routes. One is the direct action of a kinase on i^6Ado and the other is by the action of a specific purine phosphoribosyl transferase on the base, i^6Ade. It has already been proposed that for the formation of N^6-benzyladenosine monophosphate, only one of these two alternatives operates in certain plant tissues (Doree *et al.* 1972; Elliot and Murray, 1972). Although it is not known which pathway predominates in the KX and O-1 tissues, the results show that an enzyme system, capable of catalyzing the formation of these compounds, is present in both normal and autonomous tissues of the tobacco callus.

The formation of RZ from i^6Ado is catalyzed by an enzyme which hydroxylates the side chain of the cytokinin.

Muira and Hall (1973) have also shown that corn endosperm and the mycorrhizal fungus, *Rhizopogon roseolus*, contain a similar hydroxylating enzyme. Whitty and Hall (1974) extended these studies with this enzyme and have shown that all the naturally occurring cytokinins act as substrates. Hence it is assumed that the conversion of RZ into Ado is mediated by cytokinin oxidase. The transformation of RZ and i^6Ado is similar, that is, RZ has 4 immediate derivatives, Ado, RZ-5'-P, Z and i^6Ado.

The conversion of RZ to Z is obviously catalyzed by a nucleosidase. The presence of this activity has also been found in bean axes (Sondheimer and Tzou, 1973) and in the freshly prepared corn enzyme extracts (Whitty and Hall, 1974).

The formation of RZ-5'-P from RZ can be catalyzed either directly by a kinase or indirectly by a purine phosphoribosyl transferase via Z, as in the case of i^6AMP. Although the conversion of RZ into i^6Ado has not yet been reported, this possibility cannot be absolutely ruled out. This suggests that the reverse reaction of the hydroxylation of the side chain, i.e. the dehydroxylation step, could exist in the tissue.

The formation of RZ-5'-P starting from i^6Ado can follow two possible paths because the nucleotide of RZ can be formed from either RZ or i^6AMP. It is not certain whether the formation of i^6AMP or the hydroxylation of the side chain is the initial step in the conversion. The third possibility is that both pathways function under appropriate conditions. It is premature, however, to state exactly how the pathways are operating, but it is quite certain that RZ-5'-P can be obtained from i^6Ado in the tobacco callus.

In the case of i^6Ade, the immediate derivatives are Ade, Z, i^6AMP and the starting compound, i^6Ado. The formation of the Ade seems to be catalyzed by cytokinin oxidase, a reaction already studied *in vitro* using a preparation from corn (Whitty and Hall, 1974). Zeatin is formed from i^6Ade by the action of the hydroxylating enzyme. Muira and Miller (1969) were the first to detect the presence of such an enzyme in the mycorrhizal fungus, *Rhizopogon roseolus*. If the possibility of conversion of i^6Ade back to i^6Ado is included, then i^6AMP can be formed either directly by a purine phosphoribosyl transferase or indirectly via i^6Ado, as discussed in the previous section.

The other problem is the route of formation of Z from i^6Ado. This could occur via RZ or via i^6Ade, but at the moment it is impossible to predict which pathway predominates in the tobacco callus.

With respect to Z, the elimination of the side chain, i.e. the conversion of Z into Ade, is catalyzed by cytokinin oxidase. The formation of Z from both RZ and i^6Ade has been discussed above. The forward reactions are obviously functioning; the possibility of the reverse reaction, i.e. the conversion of Z back to RZ and i^6Ade, cannot be ignored. The formation of RZ-5'-P from Z can be accomplished either by the action of kinase on RZ indirectly or by a purine phosphoribosyl transferase directly on zeatin.

The evidence indicates that i^6Ado is metabolized into various products in KX and O-1 tissues. Although some uncertainties exist in the determination of the dominant pathways in the transformation of i^6Ado into its metabolites, there is no doubt that an i^6Ado metabolic network exists in both the normal and the autonomous tissues of tobacco callus. The

difference or similarity of the metabolism in these two tissues cannot be defined unequivocally at this stage of the investigation.

These findings seem to be the first report that these pathways exist together in one line of cultured tobacco callus tissue, although one or two individual pathways have been detected in different plant systems, as discussed above. Recently, the formation of a nucleotide of i^6Ado from the nucleoside in plant tissue has been reported from two laboratories (Chen *et al.* 1974; Laloue *et al.* 1974). The independent findings of these investigators are in agreement with the results reported here. It has also been reported that RZ-monophosphate can be obtained from Z in bean axes (Sondheimer and Tzou, 1971), radish seedlings (Parker *et al.* 1972) and in dormant ash embryo (Tzou *et al.* 1973). It has been shown in a number of studies that Z is not only metabolized into its ribonucleoside, ribonucleotide and even possibly the di- and tri-phosphates (Parker *et al.* 1972; Tzou *et al.* 1973), but it is also metabolized into corresponding dihydro derivatives (Sondheimer and Tzou, 1971).

The formation of inosine from i^6Ado has been shown to be catalyzed by bone marrow adenosine deaminase (Hall *et al.* 1971; Hall and Mintsioulis, 1973), another degradative enzyme similar to cytokinin oxidase in plant tissue. In addition to the above reaction, i^6Ado can be metabolized in a number of mammalian cells. Divakar and Hakala (1971) showed that in cultured sarcoma - 180 cells, i^6Ado was metabolized into various products. They found that i^6Ado has to be phosphorylated at 5'- position in order to carry out its biological function in this cell line. Although i^6Ado is phosphorylated by adenylate kinase to the 5'-monophosphate, phosphorylation could not proceed any further (Divakar

et al. 1972). Rathbone and Hall (1972) also noted that a cell line derived from human myelogenous leukemia (Roswell Park 6410) contains an enzyme that catalyzes hydrolysis of i^6Ado to the free base - i^6Ade. These results emphasize that i^6Ado can be metabolized not only in plant tissue, but also in animal cells.

Quantitative Studies

Having demonstrated the nature of metabolism of i^6Ado in normal (KX) and autonomous (0-1) tissues of tobacco callus, the quantitative relationships between metabolites were examined. Because a correlation between the intracellular level of certain synthetic cytokinins and their rate of uptake in *Acer pseudoplatanus* cells had been found (Doree and Guern, 1973; Terrine *et al.* 1972), the second part of the investigation began by studying the uptake of i^6Ado in both the KX and 0-1 tissues.

The rate of uptake of i^6Ado by KX and 0-1 tissues for the first hour of incubation period was 3.48 and 5.22 nmoles/hr/gm of fresh weight respectively (Fig. 28). Not only the rate of uptake of i^6Ado by the two tissues was different, but also the time period necessary for the 0-1 tissue to adjust to the incubation medium was shorter (Figs. 26 and 27).

The difference in i^6Ado uptake between KX and 0-1 tissues (Fig. 28) indicates that there could be some difference in cell membrane permeability of the two types of cells or a more active transporting system in 0-1 tissue. Because the 0-1 tissue is autonomous, these cells can proliferate without any exogenous plant hormone or cell division factors. Perhaps they can synthesize the necessary compounds responsible for the cell division process. This property is quite similar to that of tumor cells and therefore the 0-1 line might be considered as a cancerous-type cell.

The difference in cell membrane permeability or uptake properties between the two tissues is illustrated further in Table 6. The amount of [^{14}C] i^{6}Ado taken up by the control (KX) tissue is appreciably reduced by the addition of NAA in the incubation medium. In the case of O-1 tissue, the presence of NAA has no affect on the uptake of [^{14}C] i^{6}Ado.

In a comparative study between the normal and the crown gall tumor cells of *Vinca rosea*, it has been shown that, as a result of membrane permeability changes, tumor cells have a highly efficient ion transport system, and can grow on diluted White's medium, which does not support the growth of normal cells (Braun and Wood, 1962; Wood and Braun, 1965). A difference in cell membrane permeability or specific uptake between the tumor and normal cells has also been found in mammalian cell lines. Cooper *et al.* (1966a) demonstrated that the incorporation of [^{3}H]-TdR into TMP pool was 2 to 5 fold greater and into TDP and TTP pools was 6 to 15 times higher in chronic myelogenous leukemic cells than in normal leukocytes. As a result of the increased uptake, the incorporation of thymidine into DNA was increased in tumor cells from 13 to 87% over the concentration range of TdR tested (Cooper, *et al.* 1966b). Bremer *et al.* (1973) have shown that the uptake of [^{3}H]-cytidine and [^{3}H]-uridine and their incorporation into cellular fractions in chronic lymphocytic leukemic cells ranged from below normal to several times higher than that of normal lymphocytes.

With respect to the rate of accumulation and the rate of disappearance (or utilization) of the compounds under investigation, it seems that the metabolism of i^{6}Ado in O-1 and KX tissue is different. The pattern of the levels of various intracellular metabolites in the two

tissues was not the same after 8 hours of incubation. The decreasing order of the intracellular level of the metabolites in KX tissues was found to be i^6Ado, RZ, Z, RZ-P, i^6AMP, i^6Ade, Ade, Ado and AMP; whereas in the case of O-1 tissue, it was i^6Ado, i^6Ade, Z, RZ, RZ-P, i^6AMP, AMP, Ado and Ade (Figs. 30 and 31). The major difference was the higher relative concentration of i^6Ade in O-1 tissue but other differences are apparent. Similarly, the pattern of disappearance of these metabolites in O-1 tissue was found to be different from that of KX tissue (Figs. 32 and 33).

We conclude from these observations that the metabolism of i^6Ado in O-1 tissue is quantitatively different from that of KX tissue (Fig. 30 to 39).

It had already been shown that there are some differences in metabolic activities between the KX and O-1 tissues. Fox *et al.* (1964) showed that O_2 consumption was 35% higher in the autonomous tissue than in KX. The rate of O_2 consumption was also found to be comparable with growth rate of the tissue. The O-1 tissue grew much faster than that of the KX. They also found that there were certain qualitative differences between the utilization of glucose, because the pattern of O_2 evolution from different types of glucose, i.e. [^{14}C]-labelled at different positions of glucose, was not the same between the two tissues.

A difference in biosynthetic activity has also been noted by Einest and Skoog (1973). The natural cytokinins, i^6Ade and zeatin, could be synthesized from their immediate precursor Ade in the autonomous tissue, but this biosynthetic pathway appears to be absent or to be operating at a very low level in the normal tissue. A similar biosynthetic pathway for

the formation of arabinosyl-i^6Ade from its precursor, arabinosyl-Ade, was found in the O-1 tissue by Hall (unpublished results) (see also Introduction). Dyson and Hall (1972) found that the autonomous tissue contains about 30 nM of i^6Ado in the form of the free nucleoside, whereas the normal cytokinin dependent tissue contained only about 0.25 nM.

Hormone-Hormone Interaction

In many systems the effect of one hormone is modulated by the concentrations of other hormones. In order to study this phenomenon in KX and O-1 tissues the effect of two other hormones, NAA and ABA on the metabolism of cytokinins was examined.

The data in Tables 7 - 10 show that NAA influences the metabolism of i^6Ado in KX tissue but not in O-1. An increasing concentration of NAA reduced the uptake of i^6Ado in KX tissue but showed no effect on O-1 tissues. The interesting point to be noted here is that the ratio of the cytokinin compounds changes as the concentration of NAA was increased from 10^{-7}M to 10^{-5}M, the intracellular level of RZ-nucleotide, i^6AMP, Z and i^6Ade was reduced to about one third of the initial levels. The level of RZ, however, seemed to be unchanged under these conditions. Although the ratio of RZ with respect to i^6Ado remained unaltered, the actual intracellular level of RZ was still somewhat reduced due to the decreased level of starting compound, i^6Ado. Changes in levels of other metabolites were not significant.

These results compare with those shown in Table 6, which demonstrated the influence of NAA on the uptake of i^6Ado in KX tissue. In other words, lowering the auxin concentration in the incubation medium not only increased the uptake process, but also increased accumulation of

cytokinin compounds in KX tissue, where in O-1 tissue ratios remain unaltered.

A difference in cell membrane permeability or uptake and the intracellular level of metabolites between the two tissues appears to be yet another characteristic feature. The difference in these two properties can also be seen in the data shown in Tables 11-14. The lowering of ABA concentration correspondingly increased the ratio of certain metabolites, although the changes in ratio were not as great as those in the previous experiment. In this case the changes in ratio included certain unknown metabolites.

The results in these tables show one of the important properties of autonomous tissue. In both cases, the ratios of these metabolites in O-1 were not altered due to addition of NAA and ABA. It seems that the metabolism of an autonomous cell can not be so easily perturbed by an external agent.

In conclusion, uptake and metabolism of i^6Ado has been demonstrated. It is not clear what the rate controlling steps are in this metabolism. The results, however, will form a basis for establishing which enzymes are involved in cytokinin metabolism, their regulation and the relationship of the metabolism to the cytokinin phenomenon.

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