N^6-(Δ^2-ISOPENTENYL)ADENOSINE

THE METABOLISM AND BIOLOGICAL EFFECTS OF N^6-(Δ^2-ISOPENTENYL)ADENOSINE

by

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ABSTRACT

$\underline{N}^6$-(Δ^2-Isopentenyl)adenosine is a cytokinin, that is, it promotes cell division, growth and differentiation in certain plant tissues. Some aspects of the metabolism and biological effects of i^6Ado were studied. These studies have contributed to scientific knowledge in several areas: the biological effects of i^6Ado on animal and plant cells; the metabolism of i^6Ado by animal cells; and the origin of free cytokinins.

A. The effects of i^6Ado on a cell line derived from the circulating leucocytes of a patient with chronic myeloid leukaemia, Roswell Park Memorial Institute line 6410, were studied.

(i) The growth of 6410 cells was not stimulated by i^6Ado, but concentrations of i^6Ado of 3 $\mu\underline{M}$ inhibited the growth of this cell line. $\underline{N}^6$-(Δ^2-Isopentenyl)adenosine did not affect the duration of the lag phase of growth of these cells. There was no detectable maturation of these cells as judged by an alteration in alkaline phosphatase activity under these conditions. An increase in lactic dehydrogenase activity in 6410 cells that have been treated with i^6Ado is a non-specific effect related to the death of the cells.

(ii) $\underline{N}^6$-(Δ^2-Isopentenyl)adenosine is a component of 6410 cell tRNA in quantities approximately equal to those found in other organisms. Neither i^6Ado nor the corresponding base, i^6Ade, nor the corresponding 5' monophosphate, i^6AMP, were found free in the cells or culture medium.

(iii) The tRNA of cells cultured in the presence of i^6Ado contains a normal complement of Δ^2-isopentenyl groups. This indicates that there is no feedback mechanism between free i^6Ado and the synthesis of i^6Ado in the tRNA of these cells.

(iv) Because 6410 cells contain i^6Ado in their tRNA they are exposed to the free nucleoside that is toxic to them during tRNA turnover. However, 6410 cells contain an enzyme that converts i^6Ado to the corresponding base, that is much less toxic to these cells. This is the first time that this mechanism of detoxification has been demonstrated. The finding is of importance in the development of new cytotoxic drugs related to i^6Ado, because detoxification reactions potentially limit therapeutic value of a drug.

B. The effects of i^6Ado on a variant strain of tobacco pith tissue were studied. This is the first report of the effects of this naturally occurring cytokinin on tobacco pith callus at a cellular level.

(i) The variant strain did not differentiate to form mature shoots and leaves as does normal tissue when treated with high concentrations of i^6Ado.

(ii) Administration of i^6Ado to the variant tissue was followed by cell division and a reduction in cell size within 48 hours. After this period the cell size remained constant but the tissue continued to grow.

(iii) There was an increase in the starch content of the tissue for 10 days following the administration of i^6Ado. After this time the starch levels remained constant. This is different from the rapid fall

in starch concentrations after 12 days noted in tobacco pith tissue that forms organs.

(iv) The growth of the variant tissue was stimulated by i^6Ado. Unlike normal tobacco pith tissue i^6Ado in high concentrations, 3 μM, 9 μM, did not significantly inhibit its growth.

(v) An hypothesis is proposed relating the effects of i^6Ado energy metabolism, and tissue growth and organogenesis.

C. The biosynthesis of i^6Ade was studied using a plant pathogen Corynebacterium fascians. Cultures of this bacteria have been reported to contain up to 100 μg/litre of i^6Ade.

(i) [8 -^{14}C] Adenine and [8 -^{14}C] adenosine but not [2 -^{14}C] mevalonic acid added to cultures of C. fascians were incorporated by the bacteria. Because [2 -^{14}C] mevalonic acid was not incorporated, it could not be used as a specific label for the Δ^2-isopentenyl side-chain of i^6Ado in the tRNA of these bacteria.

(ii) [8 -^{14}C] Adenine was incorporated into free i^6Ade extracted from cultures of these bacteria. The extent of labelling of i^6Ade that was extracted when the cultures were labelled with divided doses of [8 -^{14}C] adenine throughout logarithmic growth phase, was the same as the extent of labelling that occurred when the same quantity of radio-activity was administered in one dose in late logarithmic growth phase.

(iii) When cultures of C. fascians that have been labelled with [8 -^{14}C] adenine are extracted by a procedure in which the pH never falls below 7.0, less than one fifth of labelled ^{14}C-i^6Ade is extracted compared with the amount extracted from cultures subjected to heating and mild acidification before the extraction procedure.

(iv) The quantity of i^6Ade present in the cultures was estimated to be approximately 5.0 μg/litre. Heating and acidification increased the quantity of i^6Ade present to 36 to 40 μg/litre. This increase in i^6Ade was shown to be due to release of i^6Ade from C. fascians tRNA by heating and mild acidification.

(v) Bioassay of extracts of C. fascians cultures showed that i^6Ado is only a minor component of the total cytokinin activity present. Other cytokinin activity corresponded chromatographically to i^6Ado, zeatin and ribosyl zeatin.

(vi) On this evidence, I propose that all free cytokinins are not derived directly from the corresponding cytokinin hypermodified nucleosides in the tRNA, because C. fascians tRNA has been shown to contain only one cytokinin.

tRNA may act as a store of relatively inactive cytokinins that are converted to more active cytokinins under appropriate physiological circumstances.

This would enable organisms to exert control over cytokinin activity in its tissues even in the event of wide fluctuations in the turnover of tRNA.

Inevitably, work of this nature generates new questions. An important area for future study appears to be the further definition of the metabolism of i^6Ado. It is probable that metabolites of i^6Ado could cause many of the effects that have previously been due to the parent compound. In addition, a better understanding of the mechanisms by which i^6Ado causes growth differentiation and cytotoxicity may assist in the development of new therapeutic agents.

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I should like to convey my thanks to Dr. William Dyson of the Department of Biochemistry for his valuable advice, and assistance with experiments requiring the use of gas chromatography, and to Dr. David Carr, Department of Anatomy, McMaster University, under whose direction the karyotyping of 6410 cells was done. My appreciation is extended to Miss Norma Kidd who typed this thesis and to Mrs. Barbara Bell who prepared the figures.

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INTRODUCTION

During the life cycle of an organism many complex changes occur that involve its constituent cells. These changes include cell division, growth of cells, changes in cell morphology and function, i.e. differentiation of cells, and organization of differentiating cells into organs.

Because of the undoubted complexity of these processes it would be wrong to look for a "causal agent" for one or all of them. Instead, it is better to consider agents that cause changes in the differentiated state of cells as, at best, one link in a chain of events which is repeated with each life cycle. By studying such agents it is possible that more will be learned about the continuing processes of growth, differentiation and tissue organization.

The studies described in this thesis have been done on $\underline{N}^6$-(Δ^2-isopentenyl)adenosine (i^6Ado) (Figure 1) and its corresponding base $\underline{N}^6$-(Δ^2-isopentenyl)adenine (i^6Ade) (Figure 1). These are naturally occurring members of a class of compounds that have the property of causing cell division in certain plant tissues. Such compounds are therefore known as cytokinins. These compounds have other secondary biological effects in plant and animal systems. By studying some aspects of the metabolism and biological effects of i^6Ado and i^6Ade it was hoped to learn about the processes of growth, differentiation, and tissue organization and the mechanisms that control them.

FIGURE 1

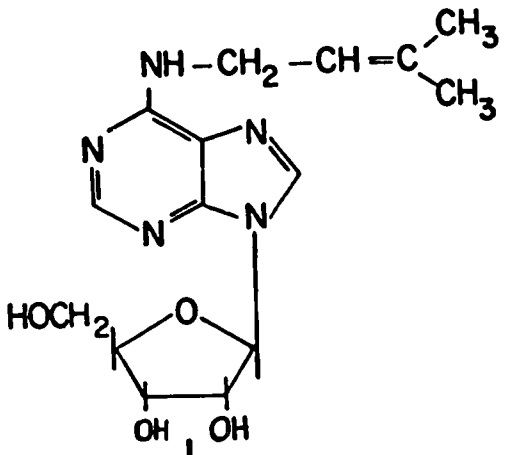
EXAMPLES OF SYNTHETIC AND NATURALLY OCCURRING CYTOKININS

1. N^6-(Δ^2-isopentenyl)adenosine, (i^6Ado) [1]
2. N^6-(Δ^2-isopentenyl)adenine, (i^6Ade) [1]
3. N^6-(cis-4-hydroxy-3-methylbut-2-enyl)adenine, (cis-zeatin) [1,2]
4. N^6-(trans-4-hydroxy-3-methylbut-2-enyl)adenine, (trans-zeatin) [1,2]
5. N^6-(Δ^2-isopentenyl)-2-methylthioadenosine [1]
6. N^6-(furfuryl)adenine [3]
7. N^6-(benzyl)adenine [3]

[1] Naturally occurring compounds

[2] Commercial samples of zeatin normally contain both cis and trans isomers in undefined proportions. The chromatographic systems described in the thesis do not separate the isomers.

[3] These are not naturally occurring compounds.



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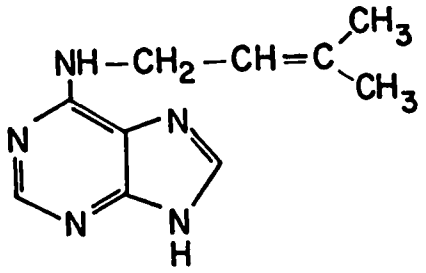

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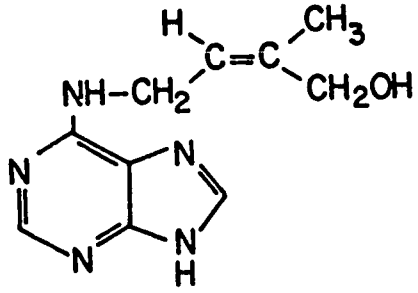

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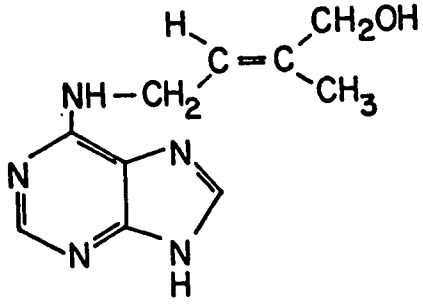

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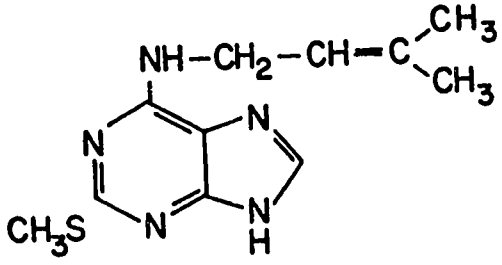

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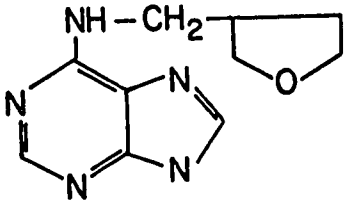

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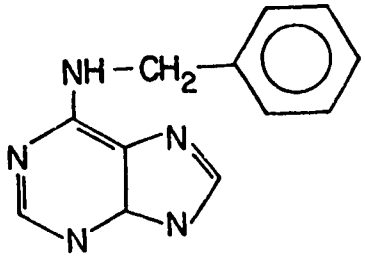

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Plant growth factors - an historical introduction

Before the turn of the century Wiesner (1892) proposed that cell division might be regulated by specific substances. Haberlandt (1913) produced the first experimental evidence for such substances. He discovered that phloem diffusates induced cell division in potato parenchyma. Later, (Haberlandt, 1921) he found that the cell division that occurs in plants after wounding was prevented by washing the wounded surface, and restored by adding crushed tissue to the wound. He named the factor that stimulated cell division "wound hormone". This substance has never been isolated or characterized.

Following these initial observations, many plant extracts were found to stimulate cell division. The most prominent of these is coconut milk, the endosperm of Cocos nucifera. van Overbeck et al. (1941) found that coconut milk stimulates cytokinesis in cultured Datura embryos.

At this time Professor Folke Skoog at the University of Wisconsin began to use plant tissues cultured in vitro to investigate the chemical control of differentiation. In 1947 he initiated cultures of stem internode segments of Nicotiana tabaccum var. Wisconsin #38. Unlike tissue cultured previously (White, 1939), stem tissue required the addition of other factors to the simple mineral medium before it would grow. Skoog and Tsui (1948) found that an auxin, such as indoleacetic acid was required to produce enlargement and proliferation of the tissue. However, when pith tissue was isolated and cultured separately from the other tissues of the stem, auxin induced only cell enlargement without cell division (Jablonski and Skoog, 1954). When vascular tissue was

placed in contact with the pith, cell division was restored in the pith. Coconut milk and malt extract (Jablonski and Skoog, 1954), yeast extract and autoclaved DNA (Miller et al., 1955a) contained activity that could promote cell division and replace plant vascular tissues in stimulating cell division. The structure of the active product from autoclaved herring sperm DNA was shown to be 6-furfurylaminopurine (Miller et al., 1955b) (Figure 1). Because of its cytokinetic activity it was named kinetin, and the generic name of kinin was proposed. Because kinin is also the term used for a class of mammalian polypeptide hormones "cytokinin" (Letham, 1963) has become accepted to designate materials with kinetin-like activities.

Although kinetin is a product of DNA - being obtained from denatured DNA - it cannot be described as a naturally occurring cytokinin, with a role in the normal physiological activities of plants. Several years after kinetin had been characterized, C. O. Miller (1962) isolated and partially characterized a naturally occurring cytokinin from immature corn kernels. Letham et al. (1964) showed this to be 6-(4-hydroxy-3-methyl-trans-2-butenylamino)purine (Figure 1). Because it was isolated from corn the substance was given the trivial name zeatin. It is the most potent cytokinin isolated to date, and has been found in a number of plant tissues (Letham, 1964; Miller and Witham, 1964).

In 1966 i^6Ado, an analogue of ribosyl zeatin, was isolated from tRNA (Hall et al., 1966; Biemann et al., 1966) (Figure 1). This compound and its corresponding base i^6Ade also have potent cytokinin activity although its activity was not so great as ribosyl zeatin and zeatin respectively (Rogozinska et al., 1964; Hall and Srivastava, 1968). Since that time,

i^6Ado or a closely related derivative have been found in all samples of tRNA investigated (Hall, 1971).

The biological activity of cytokinins

By definition, cytokinins promote cell division in some plant tissues in culture. Generally, tissues of non-meristematic origin require a cytokinin for growth *in vitro*, whereas tissues obtained from meristematic regions do not.

A commonly used bioassay for cytokinins uses this phenomenon. Cytokinin activity is determined by measuring the increase in weight, over a period of four to seven weeks, of a tissue that has an absolute requirement for cytokinin (Rogozinska *et al.*, 1964; Dyson *et al.*, 1970). In addition to this primary effect, cytokinins have a number of secondary effects on plant tissues. Many of these are not directly relevant to the work reported here, and therefore will not be discussed. However, three effects of cytokinins are relevant to this work: The Richmond-Lang effect, the control of apical dominance, and the induction of organogenesis in certain plant tissues cultured *in vitro*.

The Richmond-Lang effect

Richmond and Lang (1957) discovered that the disappearance of chlorophyll from detached *Xanthium* leaves was retarded for a number of days if the leaves were treated with cytokinin. Mothes (1960) showed that cytokinins direct the movement of numerous substances to treated areas of the leaf and prevent the movement of leaf components out of the treated area. These results suggest that the Richmond and Lang effect is in part due to the cytokinin-directed supply of nutrients to treated areas of the leaf. However, Osborne (1962) showed that

cytokinins retarded the senescence of Xanthium leaf discs even with no adjacent untreated area to act as a reservoir of nutrients. She concluded that cytokinins function by maintaining the protein synthesizing machinery. The Richmond-Lang effect has been used to bioassay cytokinin activity (Osborne and McCalla, 1961; Gunning and Barkley, 1963). There is some discrepancy between the results obtained when cytokinin activities are bioassayed using tests based on stimulation of callus growth and on the Richmond-Lang effect. Rogozinska et al. (1964) reported that the tobacco pith bioassay is relatively more sensitive to i^6Ade and related compounds than an oat leaf bioassay.

The control of apical dominance

Cytokinins counteract the usual dominance of the apical bud of plants (Wickson and Thimann, 1958; Sachs and Thimann, 1964). In fasciation disease of plants there is a loss of apical dominance with subsequent appearance of a "witch's broom" of growing shoots. This can be duplicated by treating the plant with cytokinins suggesting that these compounds are involved in the disease (Samuels, 1961). Klambt et al. (1966) and Thimann and Sachs (1966) have extracted cytokinin activity from cultures of an organism that causes fasciation disease, Corynebacterium fascians. The principle cytokinin activity in the cultures of this bacterium was subsequently identified as i^6Ade (Helgeson and Leonard, 1966).

Induction of organogenesis

Cytokinins induce organogenesis in some plant tissues cultured in vitro. This effect depends upon a balance between the auxin and cytokinin concentrations in the medium. For example, if tobacco pith

is cultured in the presence of 2 mg/litre indoleacetic acid and 0.02mg/litre kinetin the pith grows as an amorphous callus. At higher cytokinin to auxin ratios the callus differentiates to form buds, whereas at lower ratios, root formation is observed (Skoog and Miller, 1957). Only a few small areas of a callus differentiate under the influence of cytokinin. Because the differentiating cells are a distinct minority, biochemical changes taking place in them are greatly diluted by the metabolism of the remainder of the callus. This makes biochemical investigations very difficult. Therefore, most studies have used other methods, such as histochemistry, for observing the sequence of cytokinin-induced changes. Although the mechanism whereby cytokinins induce organogenesis is unknown a number of factors have been shown to be important. Examples of these are protein synthesis (La Motte, 1960; Dougall, 1962; Syono, 1965), inorganic phosphate accumulation (Skoog and Miller, 1957), lignin synthesis (Steward et al., 1958), and starch accumulation (Murashige, 1961, 1964; Thorpe and Murashige, 1968, 1970).

The starch accumulation in organ-forming pith callus in vitro is most striking (Thorpe and Murashige, 1968, 1970). These workers proposed that starch accumulation and its subsequent hydrolysis were prerequisites for the formation of Torrey's meristemoids - nests of small rapidly dividing cells that represent the initial stage of morphological differentiation of a callus. Thorpe and Murashige (1970) showed that when starch accumulation in the callus was prevented by treatment with gibberellic acid, a plant hormone that induces the hydrolytic enzyme α-amylase (Paleg, 1960a,b, 1961), no meristemoid formation occurred.

Although the role of cytokinins in inducing organogenesis is antagonized by gibberellins, Nitsch (1968) found that these two groups of hormones had synergistic effects on the growth of callus in vitro. Thus, although cytokinin-stimulated cell division occurs in the absence of starch stores, cytokinin induced organogenesis does not appear to do so. This suggests that the mechanism of cytokinin action is different in these two cases.

The effects of cytokinins on mammalian cells

In view of the potent effects of cytokinins on plants it is not surprising that several workers have investigated the effects of various cytokinins on animal systems. In 1956, Hampton et al. discovered that kinetin riboside at a concentration of 10 μM killed 99% of an in vitro culture of human fibroblasts within 24 hours. This cytokinin did not affect epithelial cell lines of human origin-HeLa, HEp#1 and HEp#2. The selective toxicity of this compound for fibroblastic cells was also demonstrated in in vitro cultures of mouse cells. Embryonic mouse skin fibroblasts were damaged more than embryonic mouse epithelial cells or mouse Sarcoma 180. The following year kinetin was reported to increase the rate of cell division in Yoshida sarcoma cells in vitro (Ogawa et al., 1957).

It was not until 1967 that the effects of a naturally occurring cytokinin, i^6Ado, on mammalian cells in vitro were reported (Grace et al., 1967). These workers showed that i^6Ado, at concentrations of 1 μg/ml, inhibited human myeloid leukaemic cells and mouse Sarcoma 180 cells. In contrast, the corresponding base, i^6Ade, at concentrations up to 50 μg/ml did not inhibit the cells. A lymphoblastic cell line, LKID, and a

line of Burkitt lymphoma cells, P-3HR-1, were not inhibited by either i^6Ado or i^6Ade. When given to a patient with promyelocytic leukaemia i^6Ado caused a clinical and haematological remission (Jones et al., 1968).

Subsequent to completion of the work on 6410 cells described in this thesis other workers have reported investigations into the effects of cytokinins on mammalian cells in vitro. Fleysher et al. (1969) demonstrated a stimulatory effect of low concentrations in the range 10^{-6} to 10^{-8} M of i^6Ado on 6410 cells but not on Sarcoma S-180 cells. A similar stimulatory effect was found when 6410 cells were treated with other N^6-substituted adenosine analogues: furfuryl, phenyl, 2-ethoxyethyl, allyl, n-propyl, isopropyl. At concentrations greater than 10^{-6} M these compounds inhibited the growth of 6410 cells.

Recently, studies by Hakala and her colleagues have suggested the mechanism whereby i^6Ado exerts is toxicity (Divekar and Hakala, 1971; Slocum et al., 1972). These workers showed that in Sarcoma S-180 cells i^6Ado is metabolized rapidly. Within 15 minutes, over 80% of [8 -^{14}C] i^6Ado was converted to the corresponding 5' monophosphate (i^6AMP). This compound was not phosphorylated further. i^6AMP inhibited adenylate kinase thus causing a shortage of ATP in the cells. As further evidence for the mechanism of toxicity of N^6-substituted adenosine analogues in Sarcoma S-180 cells, Divekar and Hakala (1971) showed that the toxicity of these compounds to Sarcoma S-180 cells is related to their ability to serve as substrates for adenosine kinase.

Other workers have investigated the effects of i^6Ado on primary cell cultures in vitro. Gallo et al. (1969) reported that i^6Ado at

concentrations of 0.1 μM stimulates DNA synthesis and mitosis in human blood lymphocytes treated with phytohaemagglutinin. At higher concentrations (10 μM) i^6Ado completely inhibits DNA synthesis and cell division in this system.

Subsequently, Pike and Dent (unpublished observations) have examined the effects of i^6Ado on sympathetic ganglia from seven day chicken embryos. i^6Ado has a Nerve Growth Factor-like effect in this system, causing the immature ganglion cells to put out neurites. Rathbone and Hall have investigated this further and found that other cytokinins also cause neurite outgrowth (unpublished observations).

Cytokinins as components of tRNA

In addition to four basic ribonucleosides, 35 modified ribonucleosides have been isolated from RNA, many of these from the tRNA. Inspection of the primary sequences of tRNA molecules reveals that they are modified at certain specific sites and that most of the modifications involve minor alterations in the structure of the bases - for example, attachment of methyl groups, replacement of an hydroxyl group by a sulphur atom or replacement of an amino group by an hydroxyl group.

There also exist "hypermodified" nucleosides. These have considerably more complex modifications than the "modified" nucleosides and are characterized by three features:

(a) a large sidechain,

(b) a functional group, such as hydroxyl or carboxyl,

(c) a location adjacent to the 3' end of the anticodon.

Some of these hypermodified nucleosides have cytokinin activity (Skoog and Armstrong, 1970). Examples of naturally occurring hypermodified nucleosides are shown in Figure 1.

N^6-(Δ^2-Isopentenyl)adenosine is the only cytokinin that has been isolated from mammalian tRNA (Skoog and Armstrong, 1970) whereas N^6-(cis-4-hydroxy-3-methylbut-2-enyl)adenosine (ribosyl zeatin) and the 2-methylthio-derivatives of this compound and of i^6Ado have also been isolated from plant sources (Burrows et al., 1971; Hecht et al., 1969).

While hydrolysates of tRNA from all species tested have cytokinin activity (Hall, 1971) the activity resides in only certain amino acid accepting species - those that respond to codons with the first base U (Armstrong et al., 1969a,b; Armstrong et al., 1970). In each case where a cytokinin has been localized, it has been situated at the 3' end of the anticodon (Staehelin et al., 1968; Zachau et al., 1966; Uziel and Gassen, 1969; Madison and Kung, 1967; Barrell and Sanger, 1969).

The location of i^6Ado in the tRNA, with its reactive allylic double bond, adjacent to the 3' end of the anticodon of certain tRNA species suggest that the hypermodified nucleoside is critical to tRNA function. This has been demonstrated experimentally.

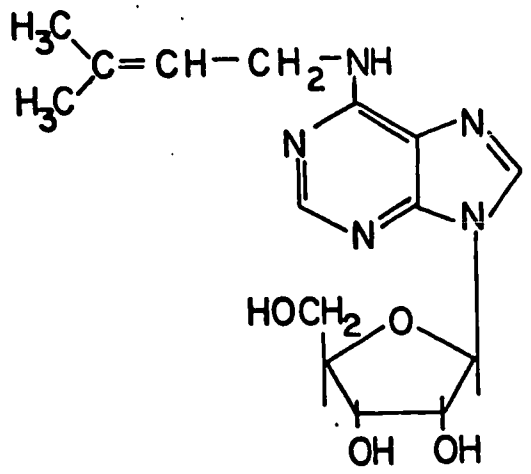
Fittler and Hall (1966) reacted i^6Ado in $tRNA^{Ser}$ with aqueous iodine to form 7,7-dimethyl-8-iodo-7,8,9-trihydropyrimido-3-(β-D-ribofuranosyl)[2,1-i]purine (Figure 2). As a result of this reaction the sidechain is fixed and an iodine atom added, which might reasonably be expected to alter the conformation of the anticodon loop. In this case the ability of the tRNA to accept serine is unaffected but its ability to bind to the messenger-ribosome complex is reduced by one-third.

Gefter and Russell (1969) isolated three species of suppressor tyrosine tRNA (Su^+III) that have identical primary sequences except

FIGURE 2

THE REACTION OF i^6Ado WITH IODINE

$\underline{N}^6$-(Δ^2-Isopentenyl)adenosine reacts with aqueous iodine at 25°C, pH 7.0 to form 7,7-dimethyl-8-iodo-7,8,9-trihydropirimido-3-(β-D-ribofuranosyl) [2,1-i]purine.



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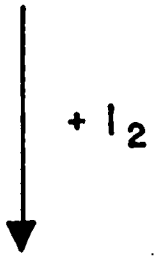
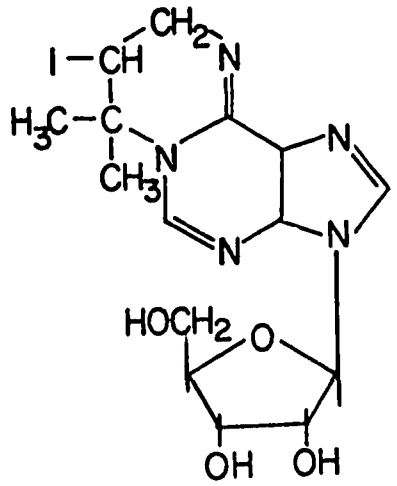




7,7-dimethyl-8-iodo-7,8,9-trihyropyrimido-3-(β-D-ribofuranosyl) [2,1-i] purine

The reaction of iodine with i^6Ado under mild conditions (25°C, pH 7·0)

that one contains $\underline{N}^6$-(Δ^2-isopentenyl)-2-methylthioadenosine, another $\underline{N}^6$-(Δ^2-isopentenyl)adenosine, and another adenosine. Whereas all species accepted tyrosine, only the first two supported protein synthesis in a system requiring suppressor tyrosine tRNA. These experiments demonstrate the importance of the Δ^2-isopentenyl group in enabling tRNA species to function.

The relationship between i^6Ado and its derivatives in the tRNA to their occurrence as free cytokinins

$\underline{N}^6$-(Δ^2-Isopentenyl)adenosine plays an important role in tRNA function. The free nucleoside and base are biologically active as cytokinins. It is important to ascertain whether there is a relationship between these two biological functions of i^6Ado. Free cytokinins and tRNA may be related in a number of ways.

(a) Cytokinins may exert some of their biological effects by becoming incorporated into the tRNA.

(b) Free cytokinins may be synthesized from adenine or a related molecule and function independent of i^6Ado in the tRNA.

(c) Free cytokinins may arise from tRNA degradation. This might be by total degradation of the tRNA molecule or by specific removal of the cytokinin moiety.

(a) There is evidence that synthetic cytokinins administered to plant tissues become incorporated into the tRNA.

Fox and Chen have studied the incorporation of the synthetic cytokinin $\underline{N}^6$-benzyladenine (Figure 1) into RNA (Fox, 1964; Fox, 1965; Fox, 1966; Fox and Chen, 1967 and 1968). These data may be interpreted as evidence of incorporation of the intact molecule into the tRNA, or in

parts following the enzymatic degradation of N^6-benzyladenine that is known to occur (McCalla et al., 1962; Guern, 1966; Fox, 1966; Dyson et al, 1972). Kinetin has been shown to be similarly incorporated into the tRNA (Wollgiehn, 1965; Srivastava, 1966).

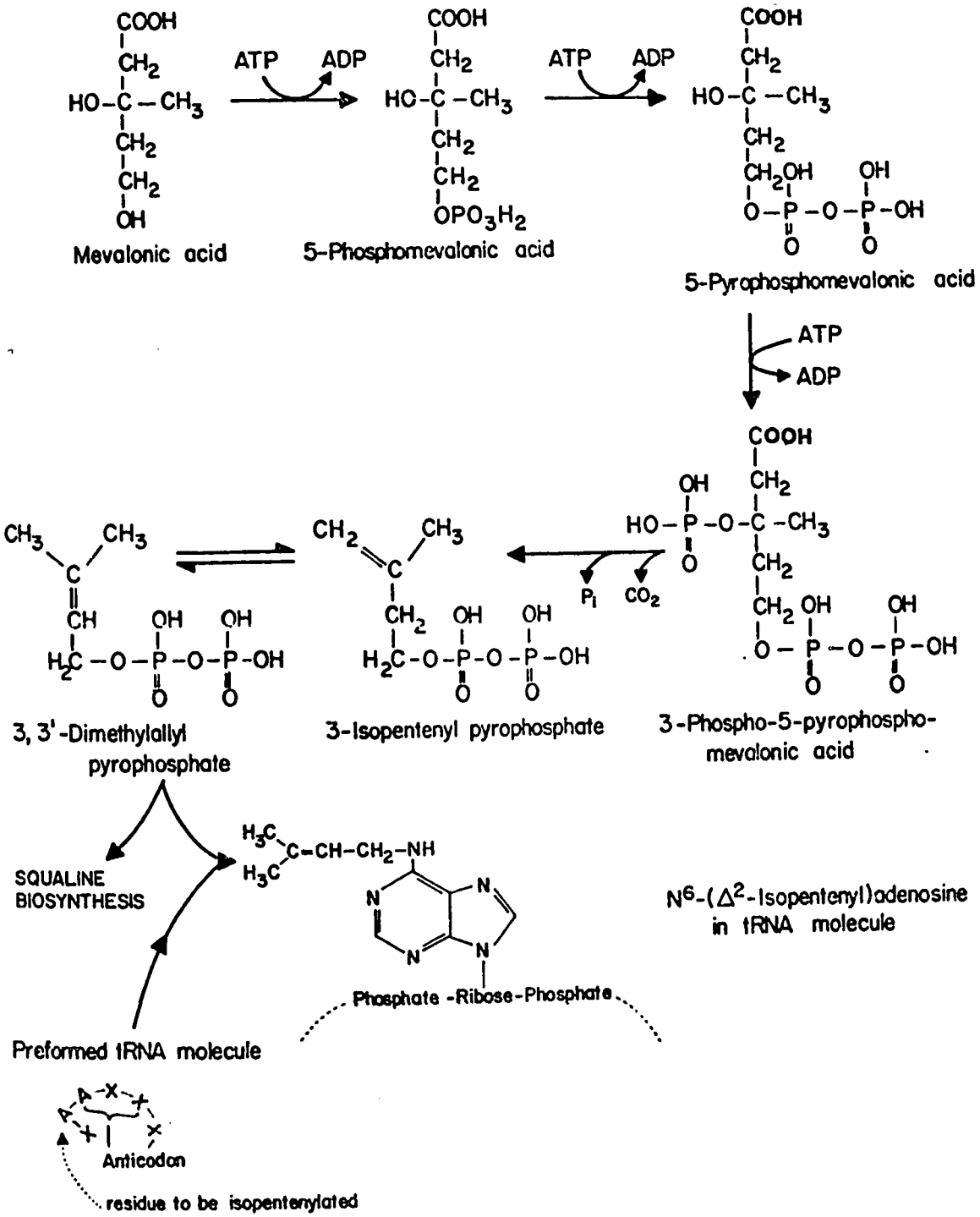
No reports exist of the effect of cytokinin incorporation on the function of the tRNA, or whether incorporation occurs at a specific site in the molecule. The physiological importance of cytokinin incorporation into the tRNA is therefore questionable.

(b) It is possible that free cytokinins are unrelated to cytokinins in the tRNA. However, all naturally occurring cytokinins that have been identified have also been isolated from samples of tRNA (Skoog and Armstrong, 1970). This suggests that there is a relationship between free cytokinins and those in the tRNA.

(c) N^6-(Δ^2-Isopentenyl)adenosine is biosynthesized in the tRNA on preformed tRNA molecules. The biosynthesis has been studied in vivo and in vitro (Fittler et al., 1968a,b; Kline et al., 1969; Chen and Hall, 1969; Peterkofsky, 1968). These workers found that the sidechain is derived from mevalonic acid via Δ^3-isopentenyl pyrophosphate and Δ^2-isopentenyl pyrophosphate. Δ^2-Isopentenyl pyrophosphate is attached to a preformed tRNA molecule by Δ^2-isopentenyl pyrophosphate: tRNA Δ^2-isopentenyl transferase. These reactions are summarized in Figure 3. This enzyme does not catalyze the incorporation of Δ^2-isopentenyl groups into tRNA from the same species. However, tRNA from E. coli B serves as a substrate for an enzyme from rat liver or yeast. tRNA from yeast and rat liver treated by permanganate to remove Δ^2-isopentenyl groups acts as a substrate for Δ^2-isopentenyl pyrophosphate: tRNA Δ^2-isopentenyl transferase from the same species. This indicates that tRNA normally contains a full complement of Δ^2-isopentenyl groups.

FIGURE 3

THE BIOSYNTHESIS OF i^6Ado IN tRNA FROM MEVALONIC ACID



The biosynthesis of i^6Ado in preformed tRNA molecules

It is possible that free cytokinins could act by suppressing the formation of the hypermodified cytokinin nucleoside in the tRNA. Hall (1970) has proposed such a mechanism and called it the "activation hypothesis". Because of the importance of the Δ^2-isopentenyl sidechain in tRNA function, tRNA molecules without the Δ^2-isopentenyl sidechain or in which the sidechain has been enzymatically modified, could be amino acylated and held in reserve in an inactive form by the cell. Attachment of the Δ^2-isopentenyl sidechain to the tRNA molecules would immediately render them active.

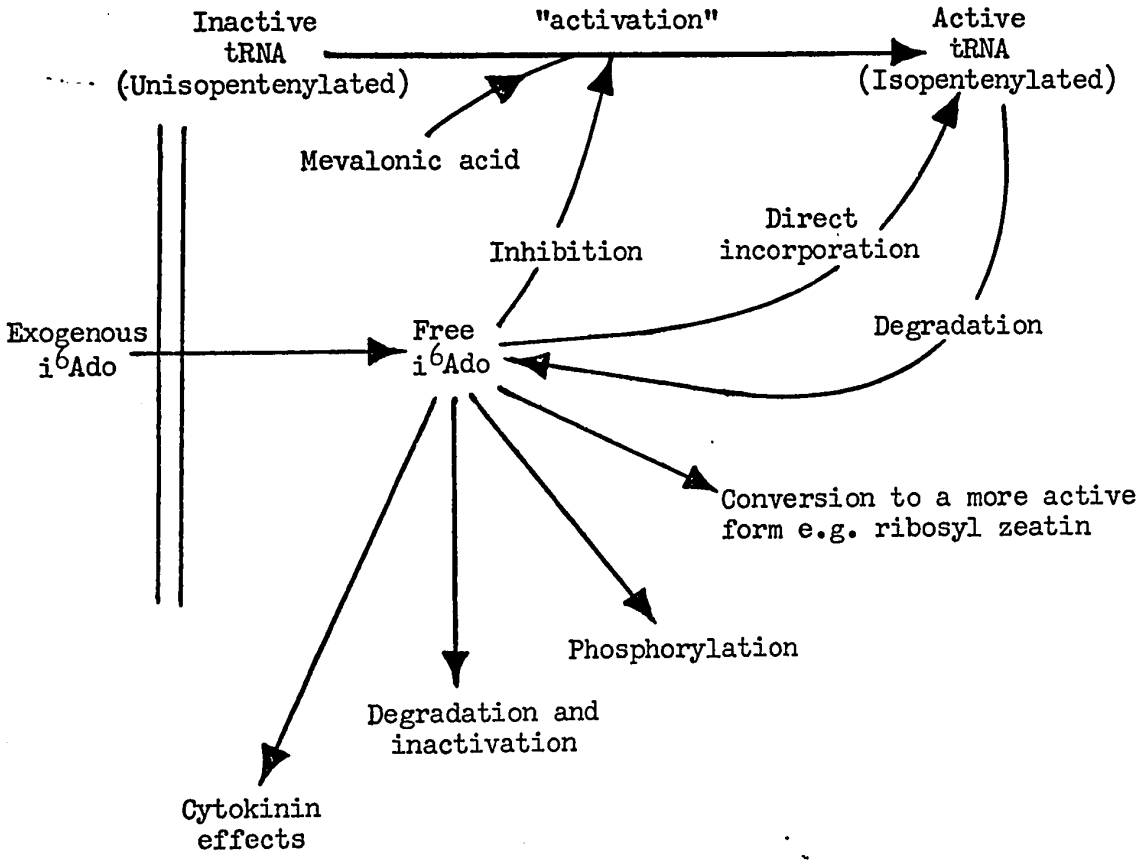
Any control of hypermodification of tRNA by free cytokinins would also affect the amount of hypermodified cytokinin nucleoside released into the cytoplasm of the cell during tRNA turnover. There is evidence for tRNA turnover in cells, for example, the tRNA of rat liver has a half-life of five days (Agarwal and Weinstein, 1970; Hanoune and Agarwal, 1970) and in humans the occurrence of modified components of tRNA in urine is evidence for tRNA turnover (Chheda, 1970).

The level of free, hypermodified nucleosides in the cells whether arising by tRNA degradation or *de novo* can also be controlled by their metabolism. N^6-(Δ^2-Isopentenyl)adenosine is degraded in plant and animal tissues (Hall and Mintsioulis, 1972; Hall *et al.*, 1971). Alternatively, the hypermodified cytokinin nucleosides may be phosphorylated. In both plant and animal systems there is evidence for both mechanisms (Slocum *et al.*, 1972; McCalla *et al.*, 1962; Guern, 1966; Dyson *et al.*, 1972).

It is also possible that i^6Ado derived from tRNA is enzymatically converted to another cytokinin. Miura and Miller (1969) found that i^6Ade was hydroxylated to form N^6-(*trans*-4-hydroxy-3-methylbut-2-enyl)adenine

(zeatin) by Rhizopogon roseolus. Some of these possible metabolic inter-relationships are summarized diagramatically below.

Some of the possible metabolic inter-relationships between i^6Ado and tRNA



Summary

Several major problems remain to be solved concerning hyper-modified cytokinin nucleosides.

The mechanism of action of cytokinins is unknown. In particular, the relationship between energy metabolism and the effects of cytokinins in causing cell growth and division and in inducing organogenesis in plants

is apparently contradictory. Although cytokinins exist in the tRNA of all species investigated (Hall, 1971) free cytokinins have only been isolated from plant and microbial sources. Our knowledge of the possible role of free cytokinins and their effects in animal systems is relatively limited compared with the knowledge of their actions in plant systems. Finally, the relationship of free cytokinins to the hypermodified cytokinin nucleosides in the tRNA in unknown. This relationship may have a considerable bearing on the biosynthesis of free cytokinins and on the control of cytokinin levels in cells.

SPECIFIC AIMS OF PRESENT STUDIES

SPECIFIC AIMS OF PRESENT STUDIES

Three major projects were undertaken in the work reported in this thesis.

(1) Leukaemic cell studies

The effects of i^6Ado on leukaemic cells *in vitro* were studied, and the findings of Grace *et al.* (1967) confirmed and extended. In particular, the effect of i^6Ado on the growth rate and maturation of RPMI 6410 cells *in vitro* was studied. I looked for the presence of i^6Ado in the tRNA of 6410 cells, and at the effect of exogenously added i^6Ado on the Δ^2-isopentenylation of the tRNA molecules, to ascertain whether a control mechanism exists between the level of exogenous i^6Ado and i^6Ado in the tRNA. Finally, I looked for evidence of a mechanism by which these cells could detoxify i^6Ado to which they would be exposed during tRNA turnover.

(2) N^6-(Δ^2-Isopentenyl)adenosine-induced differentiation in tobacco callus

Because i^6Ado has predominantly negative effects in the mammalian cell system, work was changed to a system in which i^6Ado induced readily observable positive changes - cell division and differentiation. It was hoped that the results obtained in such a system would provide a basis for later work on cytokinins in animals. Therefore tobacco pith was used to investigate i^6Ado-induced differentiation in plants. In

particular, the relationship between cytokinin stimulated cell division and differentiation was studied.

(3) The origin of free i^6Ado

One of the major questions in the cytokinin field is the mechanism of biosynthesis of free cytokinins. Initially, tobacco pith tissue was used in experiments on i^6Ado biosynthesis. However, the level of i^6Ado in the tissue proved to be too low, and therefore a third biological system was used. Corynebacterium fascians had been reported to liberate relatively large quantities of i^6Ade into its medium (Klambt et al., 1966) and thus appeared a more appropriate system to investigate the biosynthesis of i^6Ade, and its relationship to i^6Ado in the tRNA.

MATERIALS AND METHODS

MATERIALS AND METHODS

This section includes general methodology only. Specific experiments will be described in detail in the Results section.

Materials

Unless otherwise stated all chemicals and solvents were obtained from Fisher Chemical Company, Toronto. The materials were of "Certified" or "Reagent" grades. Ethanol was redistilled before use.

Biochemicals, unless otherwise stated, were obtained from Sigma Chemical Company, St. Louis. Radioisotopes were obtained from various sources as indicated in the text.

Chromatography

Chromatographic procedures of various types were used for two purposes:

(a) The purification of natural products.

(b) To identify a compound that behaves similarly to a known compound in a number of chromatographic systems.

The types of chromatography used were:

(a) Paper

(b) Thin layer

(c) Ion exchange columns

(d) Partition columns

(e) Adsorption columns

(f) Gas-liquid chromatography

Each method is discussed separately.

Solvent systems

The following solvent systems were used in the chromatographic procedures:

A. ethyl acetate:water, 4:1(v/v),

B. 1M ammonium borate, pH 9.0: 95% ethanol, 9:1(v/v),

C. 1-butanol:concentrated ammonium hydroxide:water, 86:5:14(v/v),

D. methylene chloride:methanol, 85:15(v/v),

E. benzene:methanol, 9:1(v/v),

Paper chromatography

This method was used extensively to purify materials from various biological sources, and to identify compounds. Chromatography was carried out at room temperature in a sealed glass tank. The atmosphere was saturated with the solvent used. Before use, the paper was washed with the solvent for 16 to 24 hours by descending chromatography. The paper was then washed with distilled water for 16 to 24 hours, and dried by hanging vertically in air at room temperature. This removed impurities that were soluble in the solvent and that might interfere with subsequent analytical procedures.

After the chromatograms were developed they were allowed to dry in air at room temperature, and examined under a Chromato-vue ultraviolet viewing cabinet, illuminated with short wave ultraviolet light, from a General Electric germicidal bulb, and equipped with a filter that reduces the background absorption of the paper so that very faint ultraviolet absorbing bands may be detected.

If radioactive materials were used, their positions and amounts were determined by either a chromatogram scanner (page 40) or by cutting the chromatogram into transverse strips of 1 cm width and placing each in a vial of toluene scintillation cocktail (page 41) and determining the radioactivity by liquid scintillation counting.

Thin layer chromatography (T.L.C.)

Silica gel adsorbant was used, the thickness of which was either 0.1 mm (analytical) or 1.0 mm (preparative).

The plates used for analytical purposes were obtained from the Eastman Company, Rochester, New York. They had a polyethylene tetraphthalate backing and the silica gel contained a fluorescent indicator of lead-manganese-activated calcium silicate. Before use, the plates were dried in an oven at 100°C for 30 minutes.

Preparative plates

Clean glass plates 20 cm x 20 cm were placed on a T.L.C. spreader tray, and spread with an aqueous slurry of MN-silica Gel N. There was no binder, but fluorescent indicator was used (Macherey, Nagel & Co.). The T.L.C. spreader (Desaga, Heidelberg) was set to ensure a coating of 1 mm thickness. The plates were dried in an oven at 100°C overnight and dried again at 100°C for 30 minutes before use.

Plates were developed at room temperature by ascending chromatography in a sealed tank in which the atmosphere was saturated with the solvents used. The thin layer chromatograms were dried by a stream of warm air from a hair dryer, and observed under ultraviolet light.

If radioactive material were used on the analytical plates, the silica was removed in 1 cm strips and placed in glass vials containing

toluene scintillation cocktail (page 41) and their radioactivity determined by liquid scintillation counting.

Areas from the preparative plates that contained material of interest were scraped off the plates and put into a 0.25 cm x 5 cm glass column and eluted with 95% ethanol, or 50% ethanol in water. The completeness of the elution of ultraviolet absorbing compounds was determined by examining the dried silica powder under ultraviolet light.

Ion exchange column chromatography

Preparation of DEAE cellulose columns

DEAE cellulose, an ion exchange material, was obtained from Whatman. Before use, the DEAE cellulose was stirred with 4 volumes of 1 N HCl at room temperature for 20 minutes. It was placed in a Buchner funnel and washed extensively with deionized water until the pH of the effluent was the same as that of the distilled water. The DEAE cellulose was then stirred in 4 volumes of 1 N NaOH for 20 minutes at room temperature, and washed once more with deionized water until the pH of the effluent reached that of the deionized water. The NaOH treatment was repeated and the material washed with deionized distilled water until the pH of the effluent reached that of the deionized water.

The DEAE cellulose was equilibrated by adding 0.1 M Tris-HCl, pH 7.5, and stirring gently for 15 minutes. The buffer was decanted and the process repeated eight times. The material was stored at 4°C in the buffer until required. Before use it was subjected briefly to reduced pressure in a rotary evaporator to remove dissolved gases and to prevent bubbling in the column.

DEAE cellulose prepared in this way was poured into columns. A column of 1 cm diameter x 15 cm length was sufficient to handle up to 150 A_{260} units of RNA. The columns were eluted in the manner described in the method of purification of tRNA (page 38) unless otherwise stated.

Column chromatography using Dowex 50W-X8 cation exchange resin

Dowex 50W-X8 (J.T. Baker Chemical Co., New Jersey) was made into a slurry by the addition of distilled water, and the slurry poured into a 1 cm x 15 cm glass column. This was washed (a) for 1 hour with 50 column volumes of 10% (3.0 M) NaOH, (b) for 1 hour with distilled water, (c) for 1 hour with 50 column volumes of 10% HCl and finally with (d) distilled water until the pH of the effluent was equal to that of the input.

Material was loaded onto the column in a solution adjusted to pH 3.0 by the addition of 1 N HCl. The column was washed with 1 litre of 0.3 M NaOH.

Sephadex LH-20 chromatography

Sephadex LH-20 is a bead from dextran gel prepared by hydroxypropylation of Sephadex G-25. It is designed for filtration of compounds that have a molecular weight ranging from 100 to 4000, using organic solvents. Separation may be obtained by gel filtration, adsorption or partition, depending on the solvents used and the materials to be separated.

Column preparation

Sephadex LH-20 (50 g) was prepared by swelling the material in

aqueous 35% ethanol. Excess solvent was removed and the slurry poured down a glass rod into a 2.5 cm diameter x 50 cm length column containing aqueous 35% ethanol. The aqueous 35% ethanol was drained from the column as it was replaced by the slurry. Two litres of 35% ethanol were run through the column at the rate of 30 drops/minute (50 ml/hr.) to allow the bed to settle. A disc of Whatman No. 1 paper, 2.5 cm in diameter, covered and protected the surface of the resin. The height of the resin bed was 40 cm. The column was filled to the top with solvent.

Sample application

Samples were dissolved in 2 ml of 35% ethanol. The column was run until the solvent just covered the paper disc, and the sample was applied. The column was allowed to run until the sample had entered the column and the solvent just covered the paper disc. Two ml of solvent were then added, and the column was run until the solvent again just covered the paper disc. The column was then filled with solvent and connected to a solvent reservoir. The column was eluted at the rate of 30 drops per minute, (50 ml per hour); 400 drop fractions (12 ml) were collected on an LKB "Ultrorac" Fraction Collector. The ultraviolet absorption of the eluent at 280 nm was monitored by an ISCO single beam U.V. monitor and recorded on an ISCO UA-2 Ultraviolet analyzer.

Partition chromatography using celite columns

Partition chromatography was used extensively in the early stages of the purification of cytokinins from extracts of natural materials. The method was described by Hall (1967). Acid washed Celite-545 (Johns Manville brand of diatomaceous earth) was used for all columns. The

solvent system (system A) was prepared by shaking ethyl acetate and water (4:1 v/v) in a separatory funnel until they were well mixed. The funnel was allowed to stand overnight to ensure separation of the upper phase: water saturated with ethyl acetate. Forty g of Celite were mixed with 20 ml of (aqueous) lower phase and packed into a column of 1.9 cm internal diameter, precision bore, heavy-wall tubing with a teflon ended plunger machined so that it would fit the tube exactly. The Celite-aqueous phase mixture was compressed with the plunger in small portions so that the height of each layer was equal to the internal diameter of the column. The sample was dissolved in 5 ml of lower phase solvent and mixed with 10 g of acid washed Celite. This was packed on to the top of the column in a manner similar to the other Celite. The column was developed with the upper phase of the solvent described above. The first 35 ml to elute from the column were defined as "Fraction I" and the next 100 ml as "Fraction II"; i^6Ado and i^6Ade both eluted in Fraction I.

Gas liquid chromatography (GLC)

GLC was used to assist in identifying cytokinins produced by C. fascians. Trimethylsilyl (TMS) derivatives of an extract of C. fascians culture medium that had been purified by column chromatography (page 60) were prepared. The extracts of culture medium were heated with 30 µl of N,O bis (trimethylsilyl) trifluroacetamide (BSTFA) at 65°C for 10 minutes in a reaction vessel that had a screw cap covered with teflon. An aliquot of this solution was injected into the GLC column. The column used was a 0.3 x 120 mm U-shaped glass column packed with 10% DC-11 (W/W) (Kenscott Limited, Toronto) on gas-chrom Q (Kenscott Limited,

Toronto) that had been conditioned for 48 hours before use. GLC chromatography was performed using an F and M Scientific dual column gas chromatograph, Model 402, equipped with flame-ionization detectors.

The carrier gas (helium) was allowed to flow at 35 ml/min. The column was run at a temperature of 210°C.

Control samples were prepared from i^6Ado, i^6Ade, zeatin and ribosyl zeatin. TMS derivatives were prepared from these compounds and they were subjected to GLC.

Purification of i^6Ado

Even when stored in a desiccator at -20°C, i^6Ado degrades slowly to give a number of products, and thus must be purified before use. For quantities of less than 1 mg, i^6Ado was purified by chromatography on silica gel thin layer plates, developed in 15% methanol: 85% methylene chloride (see "Thin layer chromatography" for details). The success of this method prompted development of a purification procedure for larger quantities (2 g) of i^6Ado using silica gel column chromatography. The column was prepared by adding 5% methanol: 95% methylene chloride to 40 g of silica gel (0.2 to 0.5 mm, 30 to 70 mesh A.S.T.M., Merk) to form a slurry which was poured into a 2.2 cm x 40 cm, glass column with a teflon stopcock. Two g of impure i^6Ado were dissolved in 150 ml of the 5% methanol: 95% methylene chloride solvent, and this was applied to the column. The column was monitored by spotting samples onto 0.1 mm silica gel thin layer chromatography plates, developing them in 15% methanol: 85% methylene chloride, and observing them for ultraviolet absorbing regions. The eluent contained an ultraviolet absorbing compound, Rf. 0.75.

Further elution with 7.5% methanol: 92.5% methylene chloride (600 ml) yielded i^6Ado with an Rf 0.65. Elution with 10% methanol: 90% methylene chloride (200 ml) yielded more i^6Ado, and further impurities of Rf 0.55, Rf 0.38 and Rf 0.25.

The fractions containing i^6Ado without visible impurities were pooled, dried on a rotary evaporator, recrystalized from ethanol, and lyophilized to remove traces of solvent.

Acid hydrolysis of i^6Ado and i^6Ade

When i^6Ado or i^6Ade is treated with 1 N hydrochloric acid at 100°C for 15 minutes, two products are formed as shown in Figure 4 (Hall et al., 1966).

These compounds are easily distinguished from i^6Ado and i^6Ade on the basis of their chromatographic properties. Thus, acid hydrolysis and subsequent chromatography was used as a method to provide evidence of the presence of small quantities of radioactive i^6Ado and i^6Ade in extracts of cells or medium.

Five to 10 A_{265} units of i^6Ado or i^6Ade were added to each sample as a marker. The solution was dried on a rotary evaporator, and dissolved in 1 ml of 1 N HCl. This solution was heated at 100°C on a waterbath for 15 minutes, evaporated to a small volume on a rotary evaporator, and chromatographed in one of several systems.

Extraction of RNA and purification of tRNA

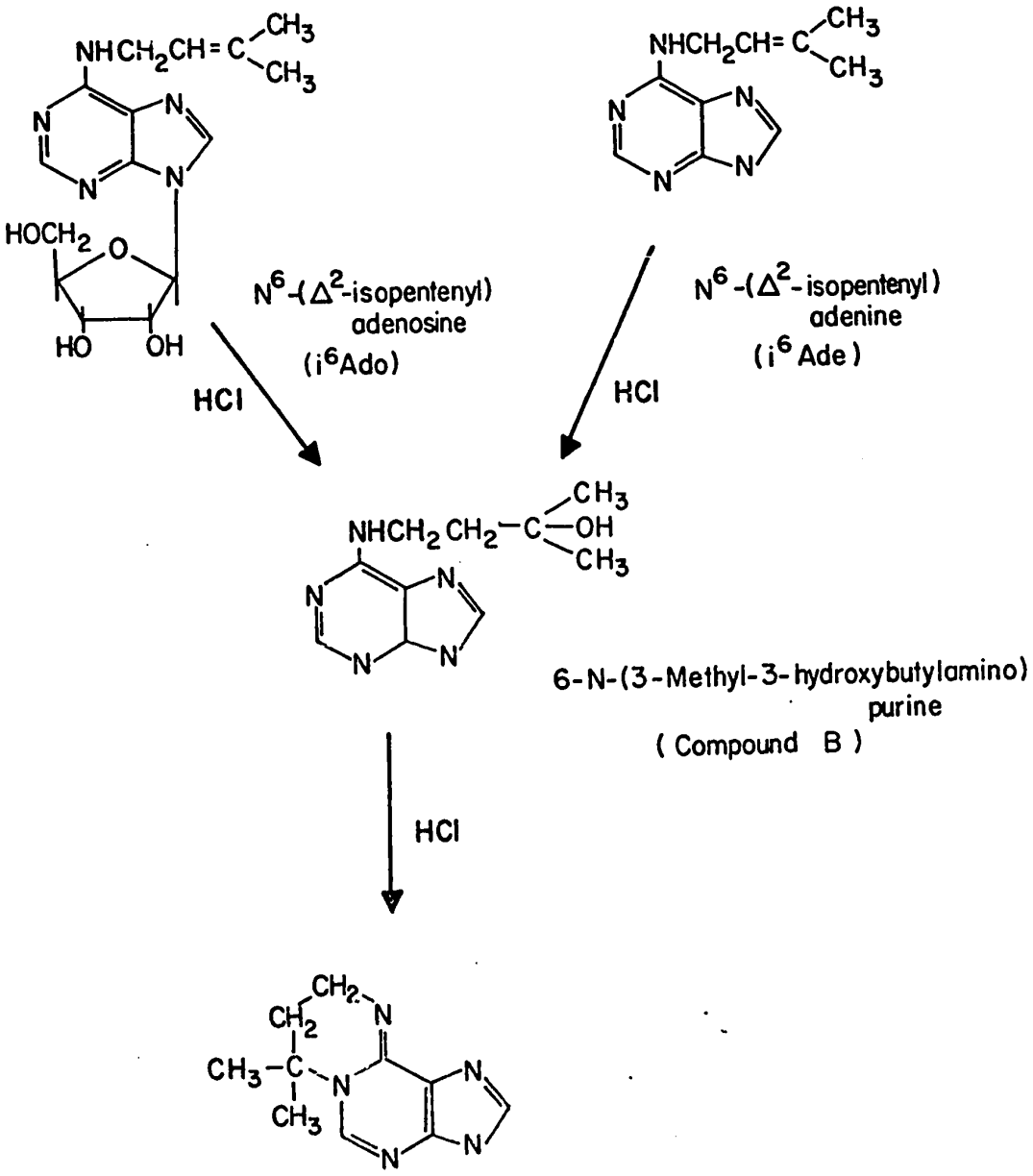
Extraction of RNA

RNA was extracted by a modification of the method described by Girard (1967). This hot phenol-sodium lauryl sulfate (SDS) technique

FIGURE 4

THE ACID HYDROLYSIS PRODUCTS OF i^6Ado AND i^6Ade

i^6Ado (1) and i^6Ade (2) hydrolysed with 1 N HCl for 15 minutes at 100°C to form product (3): 6-N-(3-methyl-3-hydroxybutylamino)purine. Under these conditions the sidechain of (3) cyclises to form: 3-H-7,7, dimethyl-7,8,9-trihydropyrimido [2,1-i]purine (4).


NHCH2CH= C CH3 CH3
HOCH2 O
HO OH
N6-(Δ2-isopentenyl) adenosine
(i6Ado)
HCl
NHCH2CH= C CH3 CH3
N6-(Δ2-isopentenyl) adenine
(i6Ade)
HCl
NHCH2 CH2 C OH CH3 CH3
6-N-(3-Methyl-3-hydroxybutylamino) purine
(Compound B)
HCl
CH2 CH2 CH3 C CH3
3-H-7,7-Dimethyl-7,8,9,- trihydropyrimido [2,1-i]-purine
(Compound A)

was first developed by Scherrer and Darnell (1962), and has been modified to take advantage of Wecker's observations (1959) on the importance of elevated temperatures (60°C) on the extraction of RNA from cells.

In these experiments cells were suspended in 5 volumes of acetate-EDTA buffer (0.01 M sodium EDTA, 0.1 M sodium acetate buffer, pH 5.1) at 0°C. 0.25 Volumes of 10% SDS were added, and mixed gently. An equal volume of 88% phenol (reagent grade) was added and the mixture was shaken in a water bath at 60°C for 3 minutes. The mixture was immediately centrifuged for 5 minutes at 5000 g at 4°C to separate the aqueous and phenolic layers. The lower (phenolic) layer was removed by suction and discarded and the upper aqueous layer and white interphase were retained. An equal volume of 88% phenol was added and the mixture was shaken for two minutes in a waterbath at 60°C. After cooling and centrifugation the phenol layer was removed and the extraction procedure repeated for a third time. The aqueous layer was then separated and retained.

Two M NaCl solution, one-tenth of the volume of the aqueous layer, and twice the volume of the aqueous layer of 95% ethanol which had been previously cooled to -20°C were added to the aqueous layer and the mixture allowed to stand at -20°C. Under these conditions RNA precipitates. The RNA was collected by centrifuging the mixture for 20 minutes at 10,000 g at 4°C and washed once with cold 95% ethanol.

Preparation of tRNA

RNA prepared by the above method was dissolved in 0.1 M Tris HCl buffer, pH 7.5. The RNA was applied to a DEAE cellulose column, (page 30) that had been equilibrated with 0.1 M Tris HCl buffer. The column was

washed once with 5 column volumes of the Tris HCl buffer, and the tRNA eluted with a gradient of NaCl (0.1 to 1.0 M) dissolved in 0.1M Tris HCl buffer, pH 7.5. The elution of the RNA from the column was monitored

(a) by observing the optical density of the eluent at 260 nm using either an ISCO UA-2 ultraviolet analyzer equipped with a monitor unit and flow cell or,

(b) by measuring the optical density of each fraction at 260 nm in a Gilford 2000 spectrophotometer using optical quartz cells of 1 cm light path.

The peak of material eluted at a NaCl concentration between 0.4 M and 0.7 M. These fractions were pooled. Two volumes of 95% ethanol that had been pre-cooled to -20°C were added to precipitate the tRNA. This mixture was allowed to stand at -20°C for 16 hours, so that further precipitation could occur. The tRNA was separated by centrifugation for 10 minutes at 10,000 g at 4°C. The ethanolic supernatant was poured off, and the tRNA that remained was either dried in a desiccator in vacuo at room temperature, or was dissolved in a small volume of the buffer that it would be used in and stored frozen at -20°C.

Hydrolysis of RNA

To isolate the component nucleosides, RNA was dissolved in 0.3 N KOH and incubated in a waterbath at 37°C for 16 hours. This alkaline hydrolysis yields 2' and 3' mononucleotides. The nucleosides were prepared from these by treating the alkaline digest with alkaline phosphatase.

The pH of the alkaline hydrolysate was adjusted to 9.2 by the addition of 1 N HCl. One mg of chicken intestinal alkaline phosphatase

(type IV, Sigma Chemical Company) was added for each 100 A_{260} units of RNA and incubated for 24 hours at 37°C. The pH of the mixture was checked after 1 hour and 3 hours incubation, and was re-adjusted to pH 9.2, if necessary.

Radioactivity determinations

Many experimental procedures used involved measurements of radioactivity. Two types of measurement were performed:

(a) the scanning of a whole paper chromatogram with a Nuclear-Chicago Actigraph III Model 1002 (Nuclear Chicago Corporation), using gas flow detectors, and

(b) liquid scintillation counting of liquid samples or of paper strips immersed in scintillation fluid. Two machines were used for this purpose: the Beckman LS-200B (Beckman Instruments Incorporated) and the Nuclear Chicago Mk I Liquid Scintillation Counter (Nuclear Chicago Corporation).

Paper chromatogram scanning

Chromatograms on Whatman I paper were cut lengthwise into strips of 1.75 cm to 5 cm width, and run vertically from the paper reel past the detectors. The radioactivity was determined by two gas flow detectors with a Micromil window (Nuclear Chicago Corporation). The start of each chromatogram was recorded on a chart recorder, having been marked on the chromatogram with a hole punch. Chromatograms were scanned at a speed of 7.5 or 15 cm per hour, with a collimator slit width of 3 or 6 mm. The Q-gas pressure was 7 psi. Q-gas was obtained from Matheson (Canada) and consisted of 1.3% butane and 98.7% helium.

Liquid scintillation counting

(a) In the Beckman LS-200B - The machine is an ambient temperature, soft beta spectrometer with 3 channels. Factory calibrated plug-in modules were plugged into each channel according to the isotopes used. The machine is automatic: counts per minute were calculated and printed out by the machine.

(b) Nuclear Chicago Mk I is a refrigerated spectrometer operating at 5°C. The machine was adjusted for counting each set of samples according to the isotopes used as in the instruction manual.

Scintillation fluid cocktails

For counting of radioactive samples the Beckman LS-200B was used with 15 ml of the following scintillation fluid cocktail per scintillation vial:

Cocktail I
5 g 2,5-Diphenyloxazole (Scintillation Grade) (Amersham Searle)
100 g Naphthalene
Dioxane (Baker Analyzed Reagent) to 1 litre

Cocktail II
5 g 2,5-Diphenyloaxazole (Scintillation Grade) (Amersham Searle)
0.1 g 1,4-bis[2-(4 Methyl-5-phenyloxazolyl)] benzene
Toluene to 1 litre

Cocktail III
4 g 2,5-Diphenyloxazole (Scintillation Grade) (Amersham Searle)
Toluene to 1 litre

The radioactivity of paper strips was counted by immersing them in 10 ml of Cocktail II when using the Nuclear Chicago Mk I, or in 10 ml of Cocktail III when using the Beckman LS-200B.

Biological systems

Three biological systems were used:

(a) 6410 human myeloid leukaemia derived cells

(b) KX-4 tobacco pith tissue

(c) Corynebacterium fascians

The 6410 line of human leukaemic cells

This line of cells was isolated at Roswell Park Memorial Institute in 1964 from a male patient suffering from chronic myeloid leukaemia. They have an appearance resembling blast cells (Iwakata and Grace, 1964). Although initially described as myeloblastic (Iwakata and Grace, 1964), these cells probably do not represent the cell type of the disease. Most cell lines initially obtained from a sample of peripheral blood have been shown to be lymphoid (George Moore, personal communication, 1970). Preliminary studies at Roswell Park Memorial Institute had indicated that i^6Ado was toxic to these cells (Grace et al., 1967).

The cells were grown in RPMI 1629 medium containing 15% foetal calf serum and GIBCO-Anti PPLO agent. All culture materials were obtained from Grand Island Biological Company - (GIBCO) - Grand Island, New York.

Preparation of culture medium (RPMI 1629)

The culture medium (Table 1) was obtained in powder form and made up in 10 litre batches. One hundred ml of GIBCO-Anti PPLO agent, 22.0 g of sodium bicarbonate, and 1.5 litre of foetal calf serum were added to the powdered medium. The mixture was made up to 10 litres by the addition of double distilled water and the pH adjusted to 7.5 with 1 N sodium hydroxide, or 1 N HCl. The medium was then sterilized by

TABLE 1

RPMI 1629 MEDIUM

This medium was developed specifically for growing human and mouse leukaemic cells at Roswell Park Memorial Institute, by Iwakata and Grace (1964).

Component	mg/l
Glucose	3000.0
$MgSO_4$-$7H_2O$	200.0
KCl	400.0
$CaCl_2$	100.0
NaH_2PO_4-H_2O	580.0
NaCl	6460.0
L-Alanine	13.4
L-Arginine (free base)	42.1 (HCl)
L-Asparagine	45.0
L-Aspartic acid	19.9
L-Cysteine	31.5
L-Glutamic acid	22.1
L-Glutamine	219.2
Glutathione (reduced)	0.5
Glycine	7.5
L-Histidine (free base)	20.9 ($HCl.H_2O$)
L-Hydroxyproline	19.7
L-Isoleucine (Allo free)	39.3
L-Leucine (Methionine free)	39.3
L-Lysine HCl	36.5
L-Methionine	14.9
L-Phenylalanine	16.5
L-Proline (Hydroxy L-Proline free)	17.3
L-Serine	26.3
L-Threonine (Allo free)	17.9
L-Tryptophane	3.1
L-Tyrosine	18.1
L-Valine	17.6
Ascorbic acid	0.5
Biotin	0.2
Vitamin B_{12}	2.0
D-Ca pantothenate	0.2
Choline Cl	5.0
Folic acid	10.0
i-Inositol	36.0
Niacin	0.5
Nicotinamide	0.5
Para-Aminobenzoic acid	1.0
Pyridoxal HCl	0.5
Pyridoxine HCl	0.5
Riboflavin	0.2
Thiamine HCl	0.2
Bactopeptone	600.0
Phenol red	10.0

filtration under an atmosphere of 5% CO_2 in air at 20 lbs per square inch pressure through a 293 mm diameter, 0.22 μ pore size Millipore filter. It was collected into sterile glass bottles under sterile glass disposal bells, in a clean environment. The medium was stored for periods of up to 1 month at 4°C.

Culture techniques

All manipulative procedures for the cell cultures were carried out using standard sterile techniques in a cabinet constructed for the purpose. When not in use, this cabinet was irradiated by an ultraviolet light that was switched off during use.

The front panel was of glass, and was raised 8 inches when in use to allow manipulations inside the cabinet.

The cells were maintained both in

(a) stationary culture, in 800 ml glass bottles containing 200 ml of culture, lying flat, in an atmosphere of 5% carbon dioxide and

(b) spinner cultures, stirred at 120 r.p.m. with a teflon coated stirring bar driven by a magnetic stirrer. The volumes of cultures in spinners varied from 50 ml to 12 litres. In all cases the cells were maintained at 37°C.

Unless otherwise stated cells were inoculated at an initial concentration of 10^5 to 2 x 10^5 per ml, and were diluted upon reaching a concentration of 10^6, just before the end of logarithmic growth phase. Usually, cells were harvested for various assays at a concentration of about 10^6 per ml.

Counting 6410 cells

6410 cells grow in clumps which vary in size from 1 cell to

several thousand cells. Before counting the cells, each culture was shaken gently. This caused a considerable reduction in clump size. One ml of cells was then removed with a sterile cotton-plugged pipette, stained with an equal volume of Trypan blue (0.1% in isotonic saline solution), gently mixed, and allowed to stand at room temperature for 5 minutes. An aliquot of the mixture was then spotted onto a counting chamber (Bright-Line Neubauer Haemacytometer - Fisher Scientific).

Because of the failure to resolve the problem of cell clumping it was only possible to count cells by means of a haemocytometer, rather than automatically by means of a Coulter Counter.

Karyotype of 6410 cells

The technique used for this procedure was that of Carr and Walker (1961). 6410 Cells growing in logarithmic phase, were harvested by centrifugation for 10 minutes at 3,000 g and resuspended in 0.9% sodium citrate solution. The hypotonic sodium citrate caused the cells to swell, and thus spread the chromosomes. After swelling, the cells were pelleted by centrifugation for 5 minutes at 1,000 g and fixed in 45% acetic acid in water. A drop of cell suspension was placed on a microscope slide and covered with a coverslip. This was placed between two pieces of paper, and pressure exerted on it by a 2.5 Kg copper rod, which was held vertically by means of two loose clamps. After the cells had been squashed in this way the slide was placed on a slab of frozen carbon dioxide and the coverslip removed with a razor blade. The slide was then passed through the following sequence:

(a) absolute ethanol for 5 minutes

(b) 0.2% parlodion (in equal parts of ethanol and ether for 10 seconds)

(c) dried in air,

(d) distilled water for 5 minutes,

(e) carbol fuchsin (for 4 minutes),

(f) two changes of absolute ethanol, the preparation being checked under a microscope until satisfactory differentiation was observed,

(g) xylene - two changes.

Permount mounting solution and a coverslip were applied. The preparations were examined and photographed using bright field illumination and a 5450 Å interference filter.

Tissue culture studies with tobacco pith tissue (KX-4 strain)

Source

The tobacco tissue strain used in this study is designated KX-4, and originated from the pith tissue of the stem of Nicotiana tabacum var. Wisconsin #38. This tissue was first cultured by Skoog and Miller (1957), and the tissue used in these experiments is a descendant of that original isolate. When first cultured this strain had an absolute requirement for a cytokinin and an auxin. Recently, however, some instability in the cytokinin requirement of this tissue has been noted. Although generally dependent on the presence of cytokinin for growth, some pieces of callus were capable of slow growth, in the absence of exogenous cytokinin, after an initial lag period of about 2 weeks. Such pieces of tissue had a characteristically friable appearance, and were always discarded from the experiments. Recently the tissue lost its capacity to complete the process of differentiation into a tobacco plant under the influence of high levels of cytokinin, but

TABLE 2

PLANT CULTURE MEDIUM - "O" AND "X" TYPES

"O" type constituent	mg/l medium
NH_4NO_3	1,000.0
KNO_3	1,000.0
$Ca(NO_3)_2 \cdot 4H_2O$	500.0
KH_2PO_4	250.0
H_3BO_3	5.0
KI	0.8
$MgSO_4 \cdot 7H_2O$	300.0
KCl	50.0
$ZnSO_4 \cdot 7H_2O$	7.5
$MnSO_4 \cdot H_2O$	5.0
Thiamine HCl	0.1
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Glycine	2.0
*Na_2Fe-EDTA	35.0
Inositol	100.0
Sucrose	30,000.0
Bacto Agar (omitted for liquid culture medium)	10,000.0
Distilled water to 1 litre.	

*made by dissolving 1 g of the disodium salt of EDTA in 50 ml of water, adding 0.724 g of $FeCl_3 \cdot 6H_2O$, and dissolving this also, then diluting the solution with water to 166 ml. The resulting solution contains about 7 mg/ml of sodium ferric ethylenediaminetetraacetic acid (Sodium ferric EDTA).

The pH of the medium is adjusted to 6.5 with 1N NaOH, and sterilized by autoclaving for 30 minutes at 121°C.

"X" type medium

This differs from "O" type by the addition of indoleacetic acid 2.0 mg/litre.

still required cytokinin for growth.

This variant of the KX-4 tissue was used to investigate the sequence of events that followed the addition of cytokinin to the tissue and led to the state of incomplete differentiation.

Culture techniques for tobacco pith tissue

The pith grew as callus on solid agar medium for 3 to 8 weeks. During this time it grew to several times its original size. It was removed from the flask and divided with a scalpel into pieces each weighing about 20 mg. These were planted in flasks containing fresh medium. All operations were carried out using sterile techniques in a clean environment provided by a Baker Edgegard laminar flow sterile hood.

Preparation of KX-4 tobacco tissue for histological examination

Some easily seen structural features of living plant cells are destroyed or badly distorted by many of the fixatives and embedding media commonly used. Thus for these studies the tissues were fixed in acrolein, and embedded in glycol methacrylate polymer, by the techniques of Feder and O'Brien (1968). The fixation and dehydration steps were carried out at 4°C. The tissues were fixed for 12 hours in a solution of 10% acrolein (Shell Chemical Corporation) and then transferred to 2-methoxyethanol for 12 hours. During this period the solvent was changed twice. The specimens were then transferred to absolute ethanol for 4 hours, to n-propanol for 4 hours, and finally to n-butanol for 4 hours.

Glycol methacrylate monomer was purified by shaking 100 ml of glycol methacrylate monomer with 4 g of activated charcoal for one hour.

The mixture was filtered through Whatman I filter paper, 4 g of fresh activated charcoal was added and the mixture shaken for 1 hour. The solution was again filtered. The first part of the filtrate was contaminated with charcoal, and was collected separately from the later filtrate. The first part of the filtrate was then refiltered and the filtrates pooled. The treated monomer was stored at 4°C in the dark until required.

The embedding mixture consisted of 92.5% purified glycol methacrylate, 0.5% (v/v) 2,2'-azobis (2-methylpropionitrile), (the polymerization initiator), and 7.0% (v/v) polyethylene glycol 400, (the plasticizer) and stored at 4°C until required. The dehydrated tissues were transferred from n-butanol to the monomer mixture at room temperature. Three changes of monomer mixture were made at 24 hour intervals. The specimen infiltrated with the monomer mixture was transferred to a #00 gelatin capsule, which was filled to the top with monomer mixture. The capsule was capped firmly to entrap as little oxygen as possible because oxygen can interfere with the polymerization process. The capsules were placed in an oven at 40°C for 24 hours to allow the mixture to polymerize. The resulting block was hardened at 60°C for 24 hours. Sections of 3 to 4 μ thickness were cut using an ultramicrotome, placed on pre-cleaned slides, and stained according to one of the following procedures.

(a) Toluidine blue

1. Sections were immersed in 0.05% toluidine blue in distilled water for 5 minutes.
2. The slides were rinsed under running water for 2 minutes,

and then briefly in distilled water.

3. The slides were shaken vigorously to remove excess water and then allowed to dry at room temperature.

4. Permount mountant was applied to the slides and then a coverslip.

(b) Periodic acid-Schiff (PAS) stain, with and without blockade

Acrolein introduces Schiff-positive groups (probably aldehyde groups) into the material during fixation. When the aldehyde blockade was not performed there was, therefore, a general background staining of many tissue elements, as well as those already stained by the specific staining. The aldehyde blockade was performed by placing the slides at room temperature for 10 minutes in a saturated solution of 2,4-dinitrophenylhydrazine in 15% acetic acid in water. The sections were then rinsed thoroughly for 30 minutes in running water to ensure that no blocking agent was carried over into the first step of the staining procedure. The staining procedure was carried out as follows:

1. The slides were placed in 1% periodic acid for 10 minutes, and then washed with running water for 10 minutes.

2. The sections were placed in Schiff's reagent for 20 minutes.

3. Three successive transfers into baths of sodium metabisulfite were performed at two minute intervals.

4. The slides were rinsed in running water for 10 minutes, dried in air at room temperature. Permount mountant and coverslips were applied.

Histological examination of the material

Initially, slides of tissue harvested at each time interval were examined for evidence of obvious qualitative changes. A series of measurements was made on untreated tissue and on tissue that had

been grown with i^6Ado in the medium.

Soluble polysaccarides stain red with PAS, and the starch grains in each cell section could therefore be counted easily. The sections were always counted from one edge to the other so that the counts represent an average across the entire tissue. For each point at least 1000 grains of starch in at least 5 different sections were counted to give the level of grains per cell.

Cell size was determined by means of a measuring graticule that was calibrated with a micrometer.

Corynebacterium fascians

The C. fascians strain used in these studies was a gift from Prof. Jean Guern, Physiologie Végétale, Sorbonne, Paris. Its pathogenicity was confirmed by experiments in Dr. Guern's laboratory. Corynebacterium fascians is a plant pathogen causing fasciation disease in a variety of dicotyledonous seedlings. This process can be mimicked by inoculating the seedlings with cytokinins. C. fascians has been reported to release cytokinins into its medium, the most potent of which is reported to be i^6Ade (Klambt et al., 1966).

The growth of C. fascians

(a) C. fascians colonies took about 2 to 3 days to become visible when incubated at 27°C. Incubation at 37°C delayed this growth to 7 or 8 days. The bacteria were normally pale yellow when first visible but were deeply pigmented after they had been growing for 7 days. Stock cultures were incubated at 27°C until visible on the plates or slants and then kept at 4°C for periods of up to 6 months before being subcultured.

(b) In liquid suspension cultures - the bacteria were grown in 1 litre conical flasks each containing 500 ml of culture medium. The cultures were aerated by shaking the flasks in a New Brunswick waterbath at 100 r.p.m. The waterbath temperature was maintained at 27°C. Growth rate was ascertained by withdrawing an aliquot of the culture with a sterile, cotton-plugged pipette and measuring the optical density of this at 540 nm in a Gilford spectrophotometer (Model 2000). The optical density of these cultures was assumed to be directly proportional to the density of bacteria in the cultures.

C. fascians growth medium

Stock cultures of C. fascians were maintained on agar slants or in petri-dishes on the defined medium shown in Table 3.

Growth media and solutions of drugs and radioisotopes used in tissue culture experiments with 6410 cells, KX-4 tissue, and Corynebacterium fascians were all sterilized either by autoclaving or by Millipore filtration.

(a) Heat stable materials

Materials or solutions known to be heat stable, such as O medium, X medium and C. fascians medium, were sterilized by autoclaving in a steam autoclave, at 121°C with slow exhaust. When the material used had a volume of less than 10 ml it was autoclaved for 10 minutes at 121°C. Volumes up to 1 litre were autoclaved for 30 minutes; for volumes up to 4 litres sterilization was carried out for 45 minutes. Millipore filters and filterholders were autoclaved under the same conditions for various periods as directed by the manufacturers.

TABLE 3

CORYNEBACTERIUM FASCIANS CULTURE MEDIUM

(After Klambt et al., 1966)

Constituent	mg/litre of medium
Sucrose	20,000
l-asparagine	2,500
KH_2PO_4	1,000
Thiamine hydrochloride	1
Bacto Agar (Difco)[1]	20,000
Trace element solution[2]	1 ml.

1. Added only to medium required for plates and slants used in routine maintenance of C. fascians stocks.

2. The trace element solution had the following composition:

Constituent	mg/litre
H_3BO_3	0.62
KI	0.083
$NaMo_4 \cdot 2H_2O$	0.025
$Co\ Cl_2 \cdot 6H_2O$	0.0025
$Mg\ SO_4 \cdot 7H_2O$	37.0
$Mn\ SO_4 \cdot 4H_2O$	2.23
$Zn\ SO_4 \cdot 7H_2O$	0.86
$Cu\ SO_4 \cdot 5H_2O$	0.0025
*Na_2Fe EDTA	3.5

* made as described in Table 2 , page 47.

(b) Heat labile materials

Heat labile materials, and materials whose stability was unknown, were sterilized by Millipore filtration. RPMI 1629 culture medium was sterilized by passage through a 293 mm or 142 mm diameter Millipore filter of 0.22 μ pore size, propelled by an atmosphere of 5% CO_2 in air (Matheson, Canada) at 20 lbs. per square inch pressure. The filtered medium was collected aseptically in sterile bottles.

Smaller volumes of material were sterilized using a 13 mm Swinnex filter (Millipore Corporation) of 0.22 μ pore size.

Enzyme assays

(a) Lactic dehydrogenase (LDH) assay

The assay method used was slightly modified from that of Wroblewski and La Due (1955) and was based upon the following reaction:

$$\underset{(\text{High } A_{340})}{\text{Pyruvate + NADH}} \xrightarrow{\text{Lactic dehydrogenase}} \underset{(\text{Low } A_{340})}{\text{Lactate + NAD}}$$

The progress of this reaction can be followed spectrophotometrically observing the change of absorbance at 340 nm.

Cells were harvested by centrifuging 10 ml aliquots of culture for 20 minutes at 20,000 g at 4°C, and resuspended to a final volume of 2 ml in a solution containing 0.05 M phosphate and 0.01 M magnesium chloride, pH 6.5. The cells were disrupted by ultra sound (Heat Systems Sonifier) for 2 periods of 30 seconds, while being cooled in an ice bath. A 0.2 ml aliquot of the cell homogenate was added to a test tube containing 0.2 mg of high purity β - nicotinamide adenine dinucleotide

(β-NADH) and 2.8 ml of 0.1 M phosphate buffer, pH 7.5, and the mixture incubated for 20 minutes at 25°C. 0.1 ml of 0.02 M sodium pyruvate in 0.1 M phosphate buffer, pH 7.5 were added and mixed with the contents of the tube, which were immediately transferred to an optical quartz cuvette of 3 ml capacity, 1 cm light path. The change in optical density at 340 nm was recorded for 3 minutes using a Gilford 2000 spectrophotometer, and distilled water as a reference blank. L.D.H. activity was calculated by determining the rate of change of optical density during the first 5 minutes of the reaction when the change was linear with time. As defined, one unit of L.D.H. activity causes a decrease in A_{340} of 0.001 units per minute at 25°C in a 3 ml reaction mixture in a cuvette of 1 cm light path.

(b) Alkaline phosphatase assays on 6410 cells

(i) Preparation of samples for alkaline phosphatase assays

A 10 ml aliquot of 6410 cells was removed from each culture using sterile techniques, and the cells were harvested by centrifuging at 20,000g for 20 minutes at 4°C. The cells were resuspended in 2 ml of distilled water, and disrupted by sonification for 2 periods of 5 seconds using a Heat Systems Sonifier. The suspensions were cooled in an ice bath during this procedure.

(ii) Alkaline phosphatase assays

Alkaline phosphatase activity of the cell homogenate was assayed using p-nitrophenyl phosphate as a substrate (Bessey et al., 1946; Fujita, 1939). 0.5 ml of (0.08 M) glycine buffer pH 10.5 and 0.5 ml of a solution of p-nitrophenyl phosphate (4 mg per ml) were pipetted into each of 2 tubes and warmed to 37°C. Into one tube 100 µl of water was

pipetted (reagent blank) and into the other 100 µl of cell homogenate was added. The tubes were then incubated at 37°C for exactly 30 minutes. The reaction was stopped and the colour developed by the addition of 3 ml of 0.02 M NaOH to each tube. The samples were transferred to optical quartz cuvettes of 1 cm light path and using the absorbance of the reagent as a reference, the absorbance of the reaction mixture was measured at 410 nm in a Gilford 2000 spectrophotometer. Concentrated HCl (0.1 ml) was then added to each tube to remove the colour due to p-nitrophenol and the absorbance recorded. This gave an index of the absorbance due to the homogenate itself. The alkaline phosphatase activity was then calculated from a calibration curve, constructed in the following manner:

0.5 ml of p-nitrophenol solution, 10 M, was mixed with 0.02 N NaOH solution and made up to 100 mls by adding the NaOH solution. To six tubes 1 ml, 2 ml, 4 ml, 6 ml, 8 ml and 10 ml of this solution was added and to each tube was added respectively 10 ml, 9 ml, 7 ml, 5 ml, 3 ml and 1 ml of 0.02 N NaOH. The contents of the tubes were mixed and the absorbance of the solutions at 410 nm determined spectrophotometrically. The absorbance of the 6 tubes were said to correspond to 1, 2, 4, 6, 8 and 10 arbitrary units of alkaline phosphatase activity.

The absorbance of the six solutions at 410 nm was plotted against the alkaline phosphatase units per ml.

The preparation of [2 -^{14}C] mevalonic acid from its dibenzylethylene-diamine (DBED) salt

The DBED salt of [2 -^{14}C] mevalonic acid was obtained from New England Nuclear Corporation (specific activity of 6.25 mCi/m mole).

The DBED salt was converted to its corresponding free acid by addition of an excess of a sodium bicarbonate solution. The free amine was extracted using ether. Residual ether was blown off and an equimolar amount of HCl was added to neutralize the aqueous solution of the sodium salt of mevalonic acid. The neutralized solution was stored at -20°C until required for use.

Assay for Δ^2-isopentenyl pyrophosphate: tRNA Δ^2-isopentenyl transferase

(a) Preparation of enzymes

(All procedures carried out at 4°C unless otherwise stated.)

6410 Cells were harvested in late logarithmic growth phase by centrifugation for 10 minutes at 3,000 g. The cells were washed with isotonic saline, and resuspended in an equal volume of buffer "A" containing:

Tris HCl	0.05 M	
$MgCl_2$	0.005 M	pH 7.5
Mercaptoethanol	0.02 M	

The cells were disrupted by a French Press under 15,000 lbs. per square inch pressure, and centrifuged for 30 minutes at 40,000 g. The ultraviolet absorption of the supernatant was measured at 260 nm and 280 nm in a spectrophotometer.

(b) Assay of transferase activity

Four ml of the supernatant obtained after the 6410 cells homogenate had been centrifuged at 40,000 g was added to an erlenmeyer flask that contained:

ATP (50 mg in 1 ml)

[2 -^{14}C] mevalonic acid (0.5 μ moles)

tRNA - 0.25 ml of a solution containing approximately 100 A_{260} units/ml

NH_4OH (2 M) to adjust the pH to 7.5

The mixture was incubated at 37°C for 30 minutes in a reciprocating waterbath. The tRNA was reisolated by DEAE cellulose chromatography. After incubation, the assays were placed on DEAE cellulose columns, (2.5 cm x 0.5 cm) (prepared as in "Chromatography" section) that had been equilibrated with 0.1 M Tris buffer, pH 7.5. The columns were washed with 0.1 M Tris HCl buffer, pH 7.5, containing 0.1 M NaCl until the optical density of the eluent dropped below 0.05 A_{260} units per ml. The tRNA was then eluted with 3 ml of Tris HCl buffer, 0.1 M, containing 1 M NaCl, pH 7.5.

Two volumes of 95% ethanol were added and the RNA precipitated for 16 hours at -20°C, collected by centrifugation at 10,000 g for 10 minutes, washed with 95% ethanol and redissolved in 5 ml of distilled water. The quantity of tRNA was determined by measuring the optical density of the material at 260 nm in a Gilford 2000 spectrophotometer, and the radioactivity of the material was measured by adding a 1 ml aliquot to a scintillation vial containing 15 ml of dioxane scintillation cocktail I (page 41), and counted in a Beckman LS-200B liquid scintillation counter.

Desalting aqueous extracts of biological materials

200 mg of activated charcoal was made into an aqueous slurry and sandwiched between 2 layers of Celite in a 3 cm diameter glass column.

The aqueous extract to be desalted was adjusted to pH 4.0 by the addition of 1.0 N acetic acid, and put on the column and washed with 300 ml of distilled water. The material was eluted with 50 ml of a mixture of 95% ethanol, distilled water, pyridine (9:9:2 v/v). The eluent was evaporated to dryness on a rotary evaporator. Traces of pyridine were removed from the material by adding 20 ml of 95% ethanol and re-evaporating the mixture.

Assay of uptake of radioactive materials by C. fascians

Aliquots of 5.0 ml of a suspension culture of C. fascians were removed and spotted onto a Millipore filter of 0.45 μ pore size, which was attached via an erlenmeyer flask to a vacuum pump. The small yellow spot of bacteria trapped on the filter (about 1 mg) was pre-washed with 10 ml of medium over a period of 20 seconds, incubated with 3 ml of medium containing a determined quantity of the radioactive material under investigation for 2 minutes, and then washed with 10 ml of medium for 20 seconds. The filter was removed, placed in a clean glass scintillation vial and dried in an oven at 50°C for 30 minutes. Ten ml of toluene cocktail III (page 41) was added to each vial which was counted in a Beckman scintillation counter, Model LS-200B.

The uptake of the following radioactive compounds was studied:

[2 -^{14}C] mevalonic acid	2.95 μCi/μM (New England Nuclear) 918,800 d.p.m. per 3 ml of incubation medium
[2 -^{14}C] adenine	50.5 μCi/μM (New England Nuclear) 369,700 d.p.m. per 3 ml of incubation medium
[2 -^{14}C] adenosine	39.5 μCi/μM (New England Nuclear) 408,800 d.p.m. per 3 ml of incubation medium.

Technique used to extract cytokinin material from cultures of Corynebacterium fascians

C. fascians contain no more free i^6Ade per unit volume inside the cells than in their culture medium (J. Guern, personal communication). The volume of the bacteria in stationary phase is only 4 ml to 6 ml/litre and thus the quantity of free i^6Ade that they contain is very small compared with the quantity present in the medium. Therefore the bacteria were separated from the medium at the beginning of the extraction procedure, by centrifugation for 10 minutes at 10,000 g at 4°C. The medium was filtered through Celite on a Buchner funnel and evaporated to approximately one sixth of its original volume on a rotary evaporator.

The medium was then treated in one of two ways, both of which gave identical results:

(a) The medium was extracted six times by shaking in a separatory funnel with an equal volume of n-butanol.

(b) The medium was extracted with methylene chloride by continuous liquid extraction for 24 hours in an extractor.

In either case, the solvent extract was taken to dryness on a rotary evaporator, and redissolved in 5 ml of water, saturated with ethyl acetate and applied to a 50 g Celite column (page 32), which was developed with ethyl acetate saturated with water. The Celite I fraction (page 33) was collected, dried on a rotary evaporator, redissolved in 1 ml of 35% ethanol in water, and put onto a 50 g LH-20 column (page 31) and eluted with 35% ethanol in water. Fractions of 400 drops were collected, and under these conditions i^6Ade eluted in fractions 35 to 41. These fractions were pooled, dried on a rotary evaporator and redissolved in 1 ml of 95% ethanol.

Bioassay of cytokinin activity

The method of Dyson *et al.* (1970) was used. Soya bean tissue that was derived from the cotyledon and that required both an auxin and a cytokinin for growth was used as the test tissue. This tissue originated in C. O. Miller's laboratory and came to us via J. E. Fox.

The tissue was maintained on X medium plus 0.5 mg of benzyladenine per litre. Pieces, (0.2 mg), were placed three to a flask on basal medium (10 ml) containing the extract to be tested. The flasks were incubated in the dark at 25°C for 25 to 50 days. The pieces of tissue were then weighed and their weights compared with those of tissue grown on X medium with no added cytokinin.

RESULTS

RESULTS

The results in this section will be divided into three major parts:

(a) The metabolism and biological effects of i^6Ado on 6410 cells.

(b) The role of i^6Ado in inducing differentiation in tobacco pith callus.

(c) The biosynthesis of i^6Ade by the plant pathogen, Corynebacterium fascians.

The metabolism and biological effects of N^6-(Δ^2-isopentenyl)adenosine on RPMI 6410 cells in vitro

Karyotype of 6410 cells

The number of chromosomes in the 6410 cells was predominantly normal, (46), but there were frequent rearrangements in their structure. Pieces of individual chromosomes were frequently missing, and were found incorporated into another chromosome. Gross chromosomal abnormalities such as polyploidy or aneuploidy were not evident although such changes might be expected when dealing with cell lines that have been cultured in vitro for a number of years. Philadelphia chromosomes, characteristic of chronic myeloid leukaemia, were not visible.

Thus, although 6410 cells contain the normal complement of chromosomes, these chromosomes are abnormal structurally.

Experiments were performed to determine the optimum conditions for growth of 6410 cells. Two variables were investigated: the effect of serum concentration on growth rate and viability; the effect of the initial concentration of cells on their subsequent growth rate and viability.

The effect of serum concentration on the growth and viability of 6410 cells in vitro

In these experiments, cells were grown in 1629 media that contained a final concentration of 0% to 75% foetal calf serum. No difference was observed in the viability of cell cultures containing 15% to 75% foetal calf serum and all grew at the same rate. Cells re-suspended in media that did not contain foetal calf serum did not increase in number and a rapid and substantial loss in viability of the cells was observed. The results of a typical experiment are shown in Figure 5. Thus, a concentration of 15% foetal calf serum was found to be the minimum required to provide satisfactory growth of 6410 cells. This concentration was used in all subsequent experiments.

The effect of the initial concentration of 6410 cells on subsequent growth and viability of the cultures

Cultures of 6410 cells grew if they were inoculated into the growth medium at a concentration of 0.8×10^5 cells per ml or more, but at concentrations below 10^5 cells per ml the proportion of dead cells present in the cultures increased considerably but the concentration of cells in the culture increased. Cultures with an initial cell concentration of 0.5×10^5 per ml or less invariably died. The duration of lag phase of culture growth - that is the period following inoculation of a

FIGURE 5

THE EFFECT OF FOETAL CALF SERUM

ON THE GROWTH OF 6410 CELLS IN VITRO

RPMI 1629 culture medium was prepared as described on page 42, except that foetal calf serum was added to final concentrations of 0%, 15%, 30%, 40%, 50%, 75% before the medium was sterilized.

Cultures of 6410 cells were grown to late logarithmic phase. The cells were harvested by centrifugation for 10 minutes at 1,000 g at room temperature. The cell pellet was resuspended in fresh medium without serum. The cell concentration was approximately 7×10^6/ml. Sterile culture bottles, each containing 190 ml of RPMI 1629 medium that had been made up using different concentrations of foetal calf serum, were each inoculated with 10 ml of 6410 cell suspension. The total cell concentration and the concentration of viable cells in each culture were determined immediately after inoculation of the cultures, and at intervals thereafter.

The concentration of viable cells in each culture is plotted as a function of time after inoculation of the cultures.

×	0% Serum
△	15% Serum
○	30% Serum
●	40% Serum
▲	75% Serum
□	75% Serum

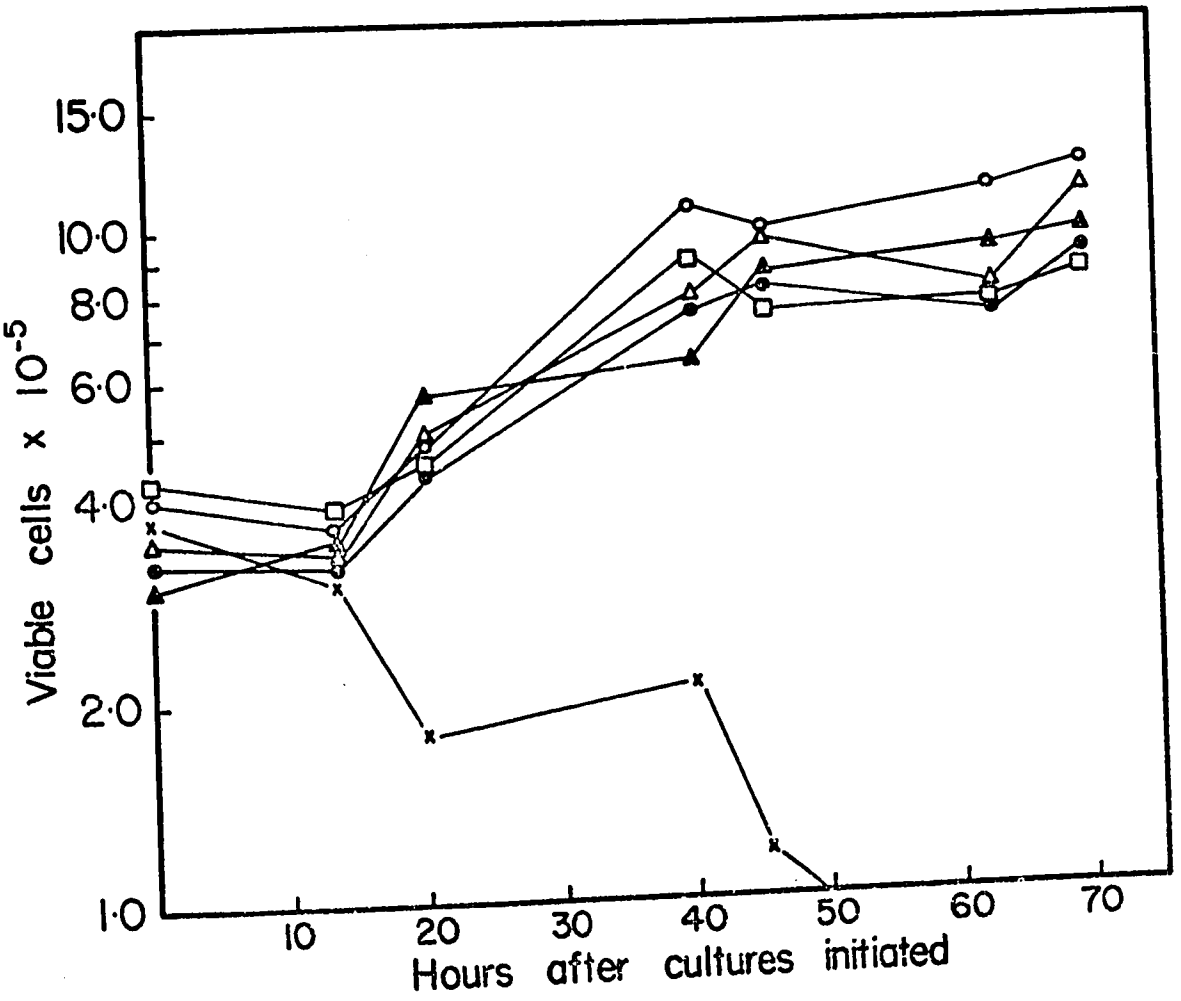

15·0
10·0
8·0
6·0
4·0
2·0
1·0
Viable cells x 10^{-5}
10
20
30
40
50
60
70
Hours after cultures initiated

FIGURE 6

THE EFFECT OF INITIAL CONCENTRATION OF 6410 CELLS ON THE SUBSEQUENT GROWTH AND VIABILITY OF CELLS IN THE CULTURES

Cultures of 6410 cells, grown to late logarithmic phase were centrifuged for 10 minutes at 1,000 g at room temperature. The cell pellet was resuspended in fresh medium containing 15% foetal calf serum. Aliquots of the cell suspension were diluted to approximately the following cell concentrations: 8×10^6/ml; 4×10^6/ml; 3×10^6/ml; 2×10^6/ml; 10^6 cells/ml. Ten ml of each dilution of cells was added to culture bottles each containing 190 ml of medium.

A. Total cell concentration as a function of time.

B. Cell viability as a function of time.

O initial cell concentration 6.4×10^5/ml

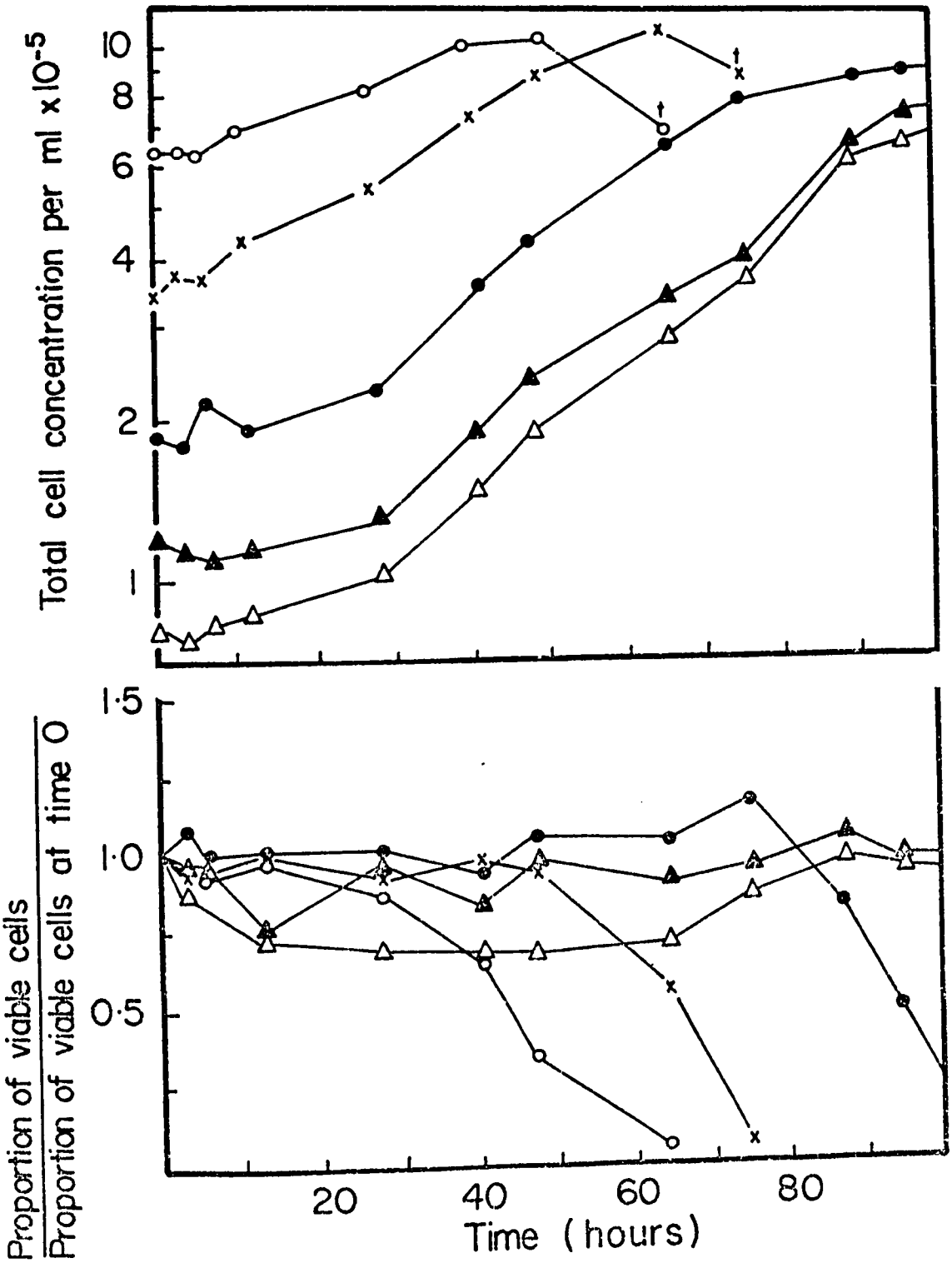
X initial cell concentration 3.6×10^5/ml

● initial cell concentration 1.9×10^5/ml

▲ initial cell concentration 1.2×10^5/ml

△ initial cell concentration 0.8×10^5/ml

† - culture viability less than 5%


Total cell concentration per ml x10⁻⁵
10
8
6
4
2
1
Proportion of viable cells / Proportion of viable cells at time 0
1·5
1·0
0·5
20
40
60
80
Time (hours)

culture before any increase in cell number was noted - depended upon the initial cell concentration (Figure 6A). When the initial concentration of cells was 10^5 per ml, there was a lag phase of 24 hours. When the initial concentration of cells was 3.6×10^5 per ml the lag phase was reduced to 6 hours. There was no lag phase when the initial cell concentration exceeded 7×10^5 per ml. The time taken for the cells in culture to double their number (doubling time) was about 24 to 26 hours and was independent of the initial cell concentration. The final concentration that was reached was also independent of the initial concentration of 6410 cells. Once the cells had reached a final concentration of about 10^6 per ml the cultures entered a stationary phase and the proportion of viable cells was less than 2% within 48 hours. Because of these findings subsequent experiments were carried out using an initial concentration of 10^5 to 3.0×10^5 6410 cells per ml. The results of a typical experiment are shown in Figure 6.

The effect of i^6Ado on 6410 cell growth and viability

The addition of i^6Ado at concentrations between 0.15 μM and 1.5 μM had no effect on the growth rate of 6410 cells in vitro (Figure 7A). In addition, the viability of 6410 cells in culture was not affected by these concentrations of i^6Ado (Figure 7B). N^6-(Δ^2-Isopentenyl)adenosine at concentrations of 3 μM or more arrested growth of the cultures and caused a progressive loss of cell viability (Figures 7A, 7B).

Similar results were obtained if i^6Ado were added when the cultures were already in logarithmic phase of growth (Figure 8). N^6-(Δ^2-Isopentenyl)adenosine did not alter the duration of lag phase of the cultures.

FIGURE 7

THE EFFECT OF i^6Ado ON THE GROWTH AND VIABILITY OF 6410 CELLS IN VITRO

Sterile aqueous solutions of i^6Ado that had concentrations of 15 μM, 30 μM, 60 μM, 150 μM were used. Two ml of each i^6Ado solution was added to a culture of 6410 cells that had a volume of 200 ml. As a control, 2 ml of sterile distilled water was added to two other cultures.

A. Total cell concentration as a function of time.

B. Cell viability as a function of time.

○	Control
⊙	Control
×	i^6Ado 0.15 μM
▲	i^6Ado 0.3 μM
▽	i^6Ado 0.6 μM
●	i^6Ado 1.5 μM
□	i^6Ado 3.0 μM
+	i^6Ado 6.0 μM

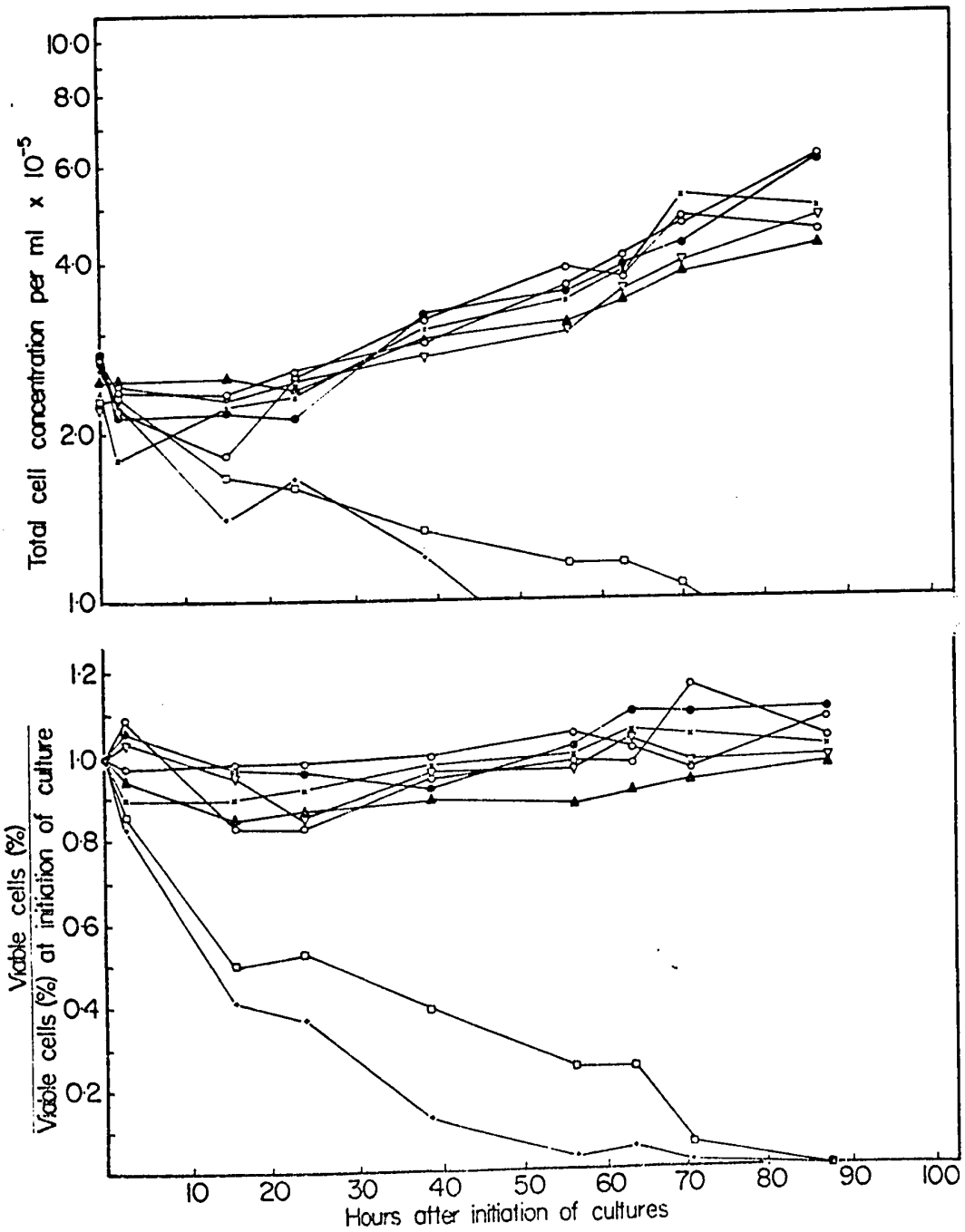

Total cell concentration per ml x 10^{-5}
10·0
8·0
6·0
4·0
2·0
1·0
Viable cells (%)
Viable cells (%) at initiation of culture
1·2
1·0
0·8
0·6
0·4
0·2
10
20
30
40
50
60
70
80
90
100
Hours after initiation of cultures

FIGURE 8

THE EFFECT OF i^6Ado ON THE GROWTH AND VIABILITY OF 6410 CELLS THAT HAVE REACHED LOGARITHMIC GROWTH PHASE

The experiment was done in a similar manner to that described in Figure 7, except that i^6Ado was added to the cultures during their logarithmic growth phase, 43 hours after they were initiated.

A. Total cell concentration as a function of time.

B. Cell viability as a function of time.

- ○ Control
- ⊙ Control
- × i^6Ado 0.15 μM
- ▲ i^6Ado 0.6 μM
- ● i^6Ado 6.0 μM

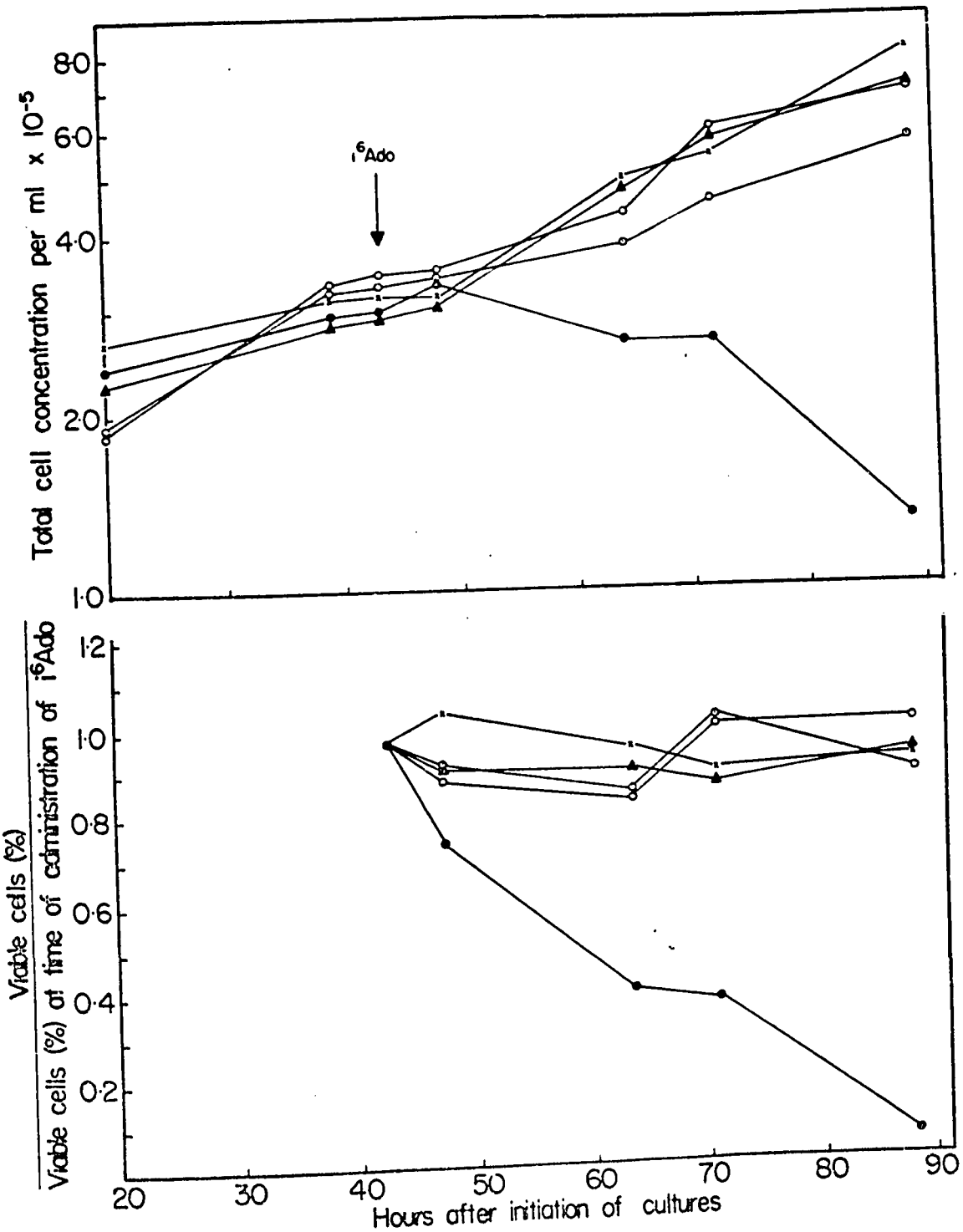

Total cell concentration per ml x 10^{-5}
8·0
6·0
4·0
2·0
1·0
i^6Ado
Viable cells (%)
Viable cells (%) at time of administration of i^6Ado
1·2
1·0
0·8
0·6
0·4
0·2
20
30
40
50
60
70
80
90
Hours after initiation of cultures

FIGURE 9

ALKALINE PHOSPHATASE ACTIVITY OF
6410 CELLS TREATED WITH i^6Ado

In these experiments, cultures of 6410 cells, each with a volume of 200 ml, were grown until they reached logarithmic growth phase. One ml of a solution of i^6Ado was added to the experimental cultures so that the final concentration of i^6Ado was 0.15 μM, 0.6μM and 3μM. One ml of distilled water was added to the control cultures. The total cell concentration, viability, and alkaline phosphatase activity, were determined at this time, and at subsequent times.

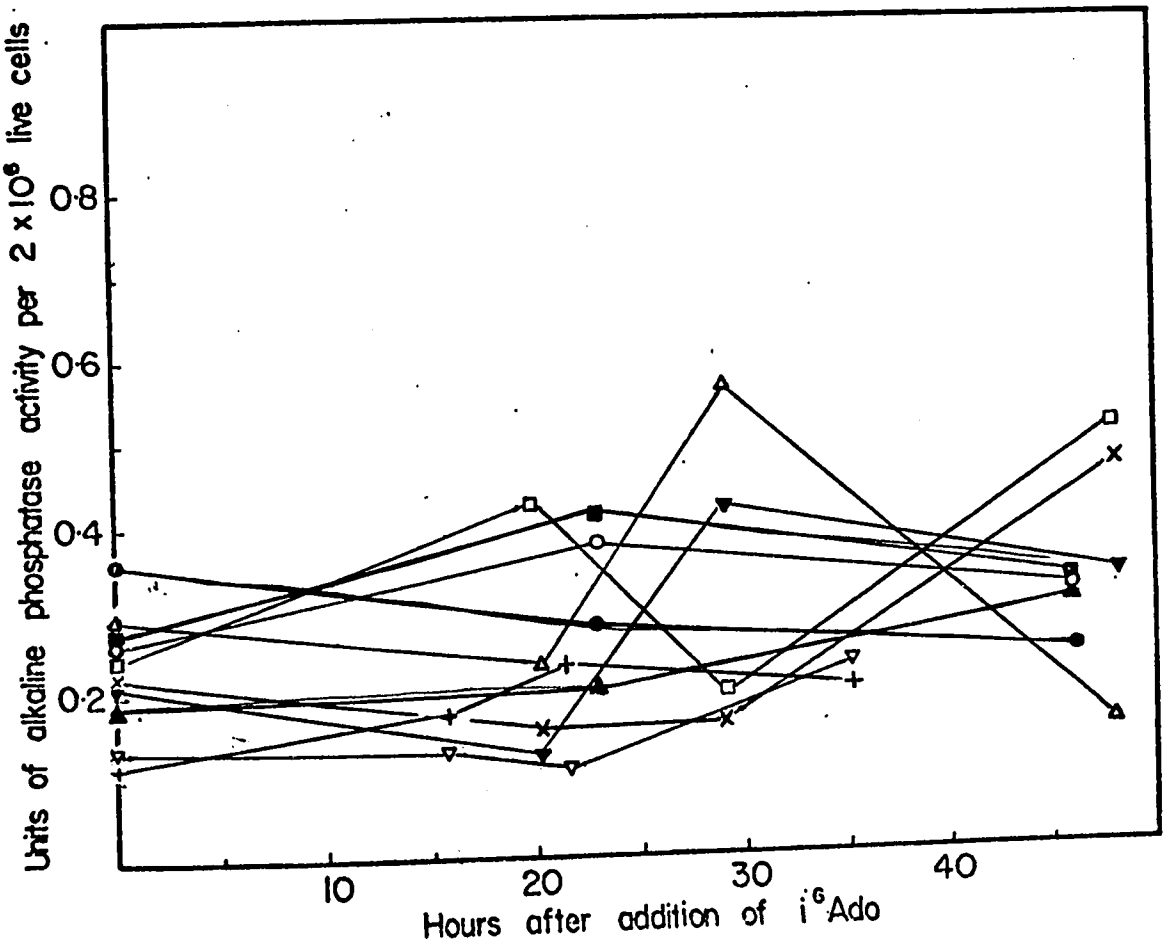
The alkaline phosphatase activity is plotted as a function of time. The figure shows the results of 3 separate experiments.

○ □ ▽ Controls

● + △ i^6Ado 0.15 μM

■ × i^6Ado 0.6 μM

▲ ▼ i^6Ado 3.0 μM


Units of alkaline phosphatase activity per 2 x 10⁶ live cells
0·8
0·6
0·4
0·2
10
20
30
40
Hours after addition of i⁶Ado

The effect of i^6Ado on alkaline phosphatase activity of 6410 cells

Neurotrophil alkaline phosphatase is reduced or absent in chronic myeloid leukaemia, although it returns to normal during remissions (de Gruchy, 1970). N^6-(Δ^2-Isopentenyl)adenosine has been reported to induce remissions in myeloid leukaemia (Grace et al., 1967) and to be toxic in 6410 cells that were obtained from a patient with chronic myeloid leukaemia (Grace et al., 1967). Therefore, the effect of i^6Ado on the alkaline phosphatase activity of 6410 cells was investigated.

Cultures of 6410 cells in logarithmic growth phase were grown in the presence of i^6Ado at concentrations between 0.15 μM and 3 μM for various periods. The alkaline phosphatase activity of the cells was determined at intervals. Because the concentrations of cells in each culture changed during each experiment the alkaline phosphatase levels per 2 x 10^6 viable cells was calculated. The results of 3 experiments are shown in Figure 9. The alkaline phosphatase activity did not change during the experiment. I concluded that i^6Ado has no influence on the alkaline phosphatase activity of 6410 cells even at concentrations that were toxic to the cells.

The effect of i^6Ado on lactic dehydrogenase (LDH) activity of 6410 cells in vitro

It has been shown (J. Blakeslee, personal communication) that when 6410 cells are treated with 0.6 μM i^6Ado there is an increase in LDH, measured histochemically. A quantitative biochemical investigation of this phenomenon was made. Cells from cultures grown without any alteration in cell viability retained a constant LDH activity per cell

FIGURE 10

LACTIC DEHYDROGENASE (LDH) ACTIVITY OF 6410 CELLS TREATED WITH i^6Ado IN VITRO

A. A culture of 6410 cells was treated with i^6Ado (0.6 μM): another culture without i^6Ado was a control. The cell concentration, viability and LDH activity were determined immediately after the addition of i^6Ado, and 12, 20 and 38 hours later. The viability of the cultures remained constant throughout the experiment.

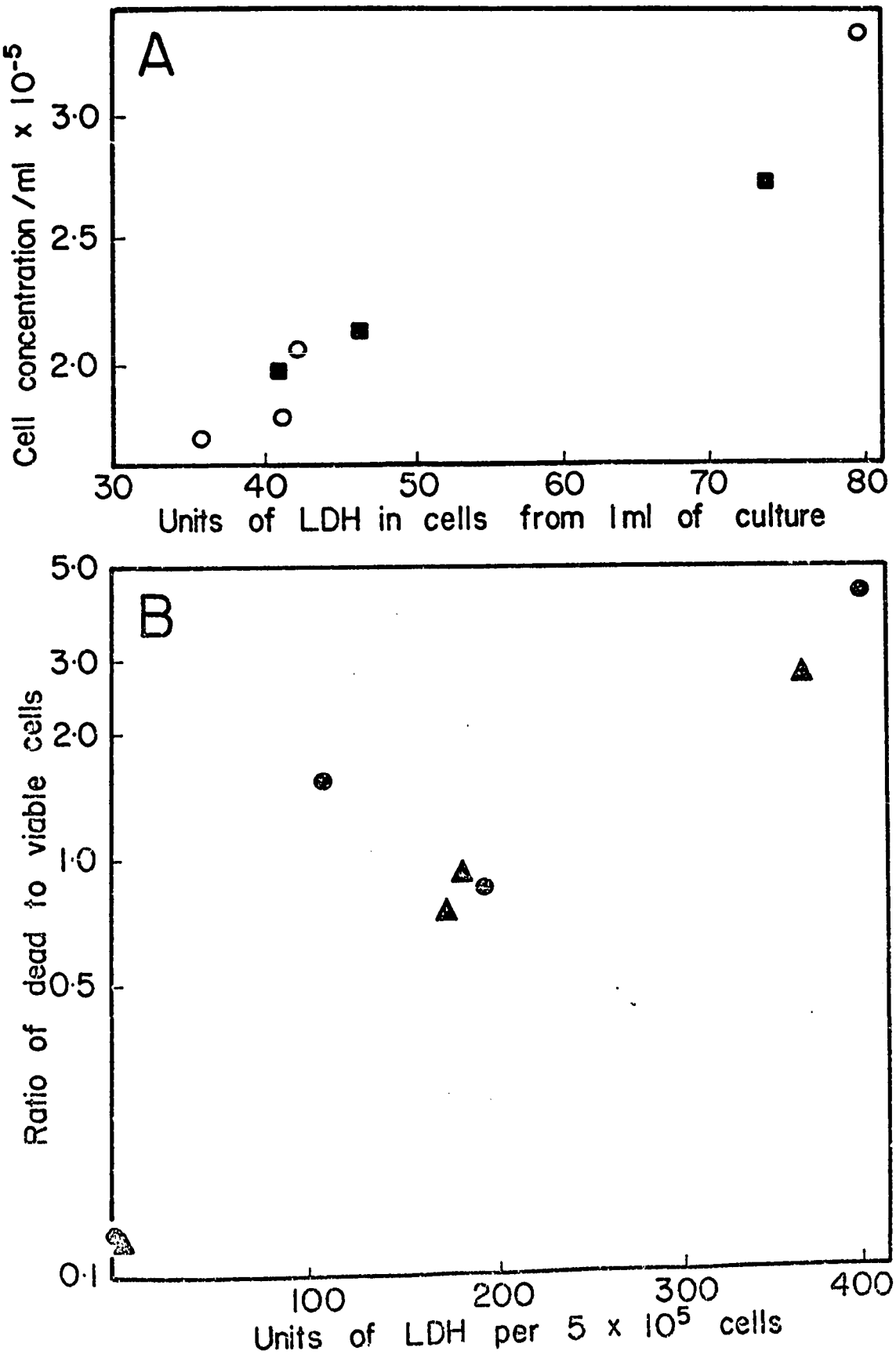
○ Control

■ i^6Ado 0.6 μM

B. A similar experimental protocol to A, except that the cells were treated with 0.3 μM i^6Ado, and the cell concentration, viability and LDH activity were determined 0, 6, 12 and 20 hours after the addition of i^6Ado. The viability of both cultures decreased but the total cell concentration did not change during the course of the experiment.

● Control

▲ i^6Ado 0.3 μM


A
Cell concentration /ml x 10⁻⁵
3·0
2·5
2·0
30
40
50
60
70
80
Units of LDH in cells from 1ml of culture
B
Ratio of dead to viable cells
5·0
3·0
2·0
1·0
0·5
0·1
100
200
300
400
Units of LDH per 5 x 10⁵ cells

(Figure10A). The addition of 0.6 μM i^6Ado, a concentration that is insufficient to kill the cells or to retard their growth, did not change the LDH activity of the cells from the control sample that did not contain i^6Ado. In some cultures there was a considerable loss of cell viability in both experimental or control cultures. This phenomenon is not uncommon in mammalian tissue culture work and has been attributed to a variety of causes. These include improper control of the pH of the medium, rough handling of the cells, or faulty rinsing of tissue culture bottles, resulting in traces of toxic materials remaining on the glass surfaces. This provided an opportunity to observe the relationship between the viability of the cultures and their LDH activity. Over the relatively short course of the experiment (20 hours) there was little change in total cell count, although the viability of the cultures (determined by trypan blue staining) was considerably reduced. The LDH activity per cell increased as the viability of the cells decreased. Thus, although cells from cultures with low viability showed an increased LDH activity, this was independent of the presence of i^6Ado, at the concentrations that were tested (Figure10B).

These results indicate that loss of viability of 6410 cells and not incubation with i^6Ado causes an increase in 6410 cell LDH activity. These data are contrary to the findings of Blakeslee, described above.

The presence of i^6Ado in the RNA of 6410 cells

N^6-(Δ^2-Isopentenyl)adenosine has been reported to be a constituent of the RNA of a number of organisms. The only location in which it has been detected is adjacent to the first base of the anticodon of tRNA species that interact with codons that have uridine in their first

position (Hall, 1971). Because 6410 cells were killed by addition of i^6Ado to the growth medium in concentrations as low as 3 μM, it was important to determine whether these cells contained i^6Ado, either as a component of their tRNA, or as a free nucleoside. If i^6Ado were indeed a normal constituent of these cells, it was possible that they could be killed by low concentrations of this normal cell constituent.

A culture of 6410 cells (3 litres) was grown to early logarithmic phase, and labelled with 50 μCi of [8 -^{14}C] adenosine (specific activity 52.6 μCi/μ mole, Schwarz Bioresearch). After 48 hours the cells were harvested by centrifugation for 10 minutes at 10,000 g at 4°C, and RNA prepared from them (page 35). The RNA was dissolved in 3 ml of distilled water. The solution was made 1 M with respect to sodium chloride and kept at 4°C for 16 hours to allow the high molecular weight RNA to precipitate. The precipitate was removed by centrifugation for 5 minutes at 10,000 g. The soluble RNA species in the supernatant were precipitated by the addition of 2 volumes of 95% ethanol at 0°C. The RNA precipitate that was insoluble in 1 M sodium chloride was redissolved in 3 ml of distilled water and precipitated by the addition of 2 volumes of ethanol at 0°C. RNA was removed from the ethanolic supernatant by centrifugation for 5 minutes at 10,000 g.

The RNA fractions were hydrolysed with KOH and treated with alkaline phosphatase to yield the nucleosides (page 39). The nucleosides were dissolved in water saturated with ethyl acetate and chromatographed on Celite columns (1.9 cm diameter x 50 cm length), developed with solvent A (page 33). The first 35 ml, and subsequent 25 ml fractions were collected. Only the first fraction contained radioactive materials

FIGURE 11

PAPER CHROMATOGRAPHY OF PUTATIVE i^6Ado

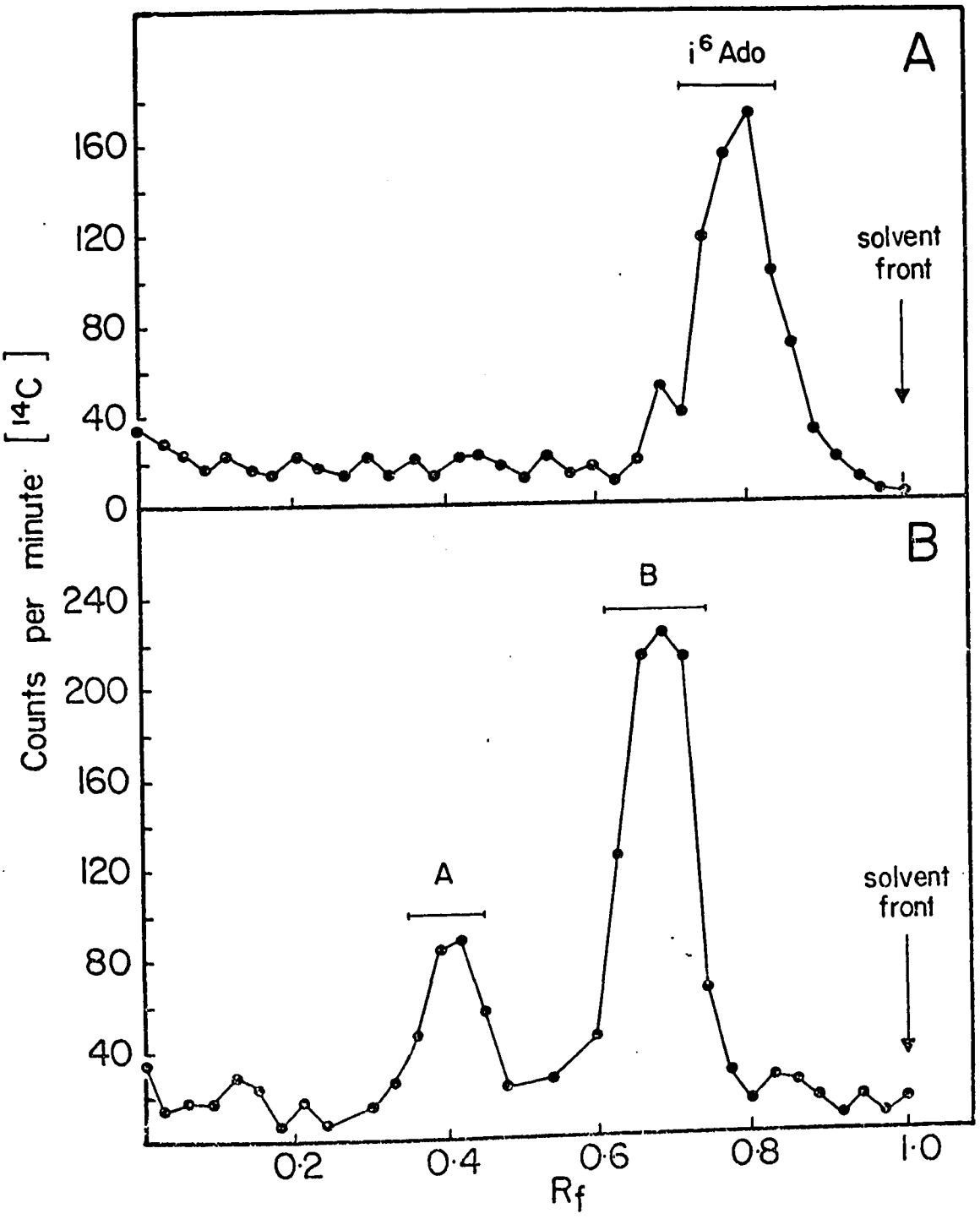
FROM 6410 CELL tRNA

Nucleosides prepared from the tRNA of 6410 cells that had been labelled with [8 -^{14}C] adenosine as described in the text, were chromatographed on a Celite column developed with solvent A (page 32).

A. Material from the fraction that contained i^6Ado rechromatographed on paper in solvent C. The chromatogram was cut into 1 cm strips, and the radioactivity of each strip determined by liquid scintillation counting.

B. Material from the fraction that contained i^6Ado, was hydrolysed with 1 N HCl (page 35) and chromatographed on paper in solvent C. The chromatogram was cut into 1 cm strips, and the radioactivity of each strip determined by liquid scintillation counting.

A,B. Position of acid hydrolysis products of i^6Ado: 3H-7,7-dimethyl-7,8,9-trihydropyrimido-[2,1-i]purine and N^6-(3-hydroxy-3-methylbutyl) adenine.


A
i⁶Ado
solvent front
160
120
80
40
0
B
B
A
solvent front
240
200
160
120
80
40
Counts per minute [¹⁴C]
0·2
0·4
0·6
0·8
1·0
Rf

that cochromatographed with i^6Ado on paper in solvent C. Upon acid hydrolysis this material yielded two products that cochromatographed with the acid hydrolysis products of i^6Ado (page 35) on paper, developed in solvent C. This demonstrated the presence of i^6Ado in 6410 cell RNA (Figure 11).

No i^6Ado was found in RNA soluble in 1 M NaCl.

On the basis of the radioactivity data it was estimated that i^6Ado comprises approximately 0.34% of the total bases in 6410 cell tRNA. This figure is in close agreement with the level found in tRNA from other sources (Robins et al., 1967).

The presence of free i^6Ado in 6410 cells

N^6-(Δ^2-Isopentenyl)adenosine, and similar compounds such as i^6Ade, zeatin and ribosyl zeatin have been found free in plant tissues (Miller, 1961; Letham and Miller, 1965; Dyson and Hall, in press), and bacterial cultures (Klambt et al., 1966; Helgeson and Leonard, 1966). There has been no report of any compound of that type being found free in mammalian tissues, despite the fact that mammalian tissues contain i^6Ado in their tRNA (Robins et al., 1967).

Therefore, experiments were done to determine if free i^6Ado were present in 6410 cells.

[8 -^{14}C] Adenosine, 50 μCi, (specific activity 52.6 μCi/μ mole, Schwarz Bioresearch) was added to a culture of 6410 cells (4 litres) in early logarithmic growth phase. After 48 hours the cells were harvested by centrifugation for 10 minutes at 3,000 g at 4°C. The cells were non-resuspended in 10 ml of 0.01 M tris buffer, pH 7.5. A sample of non-radioactive i^6Ado (0.25 mg) was added. The cells were disrupted by

sonification and an equal volume of ice cold 10% perchloric acid was added to precipitate the macromolecules. The precipitate was removed by centrifugation for 10 minutes at 30,000 g at 0°C, and the supernatant neutralized with 3 M potassium hydroxide and stored at 0°C for 16 hours. The precipitate of potassium perchlorate was removed by centrifugation for 5 minutes at 10,000 g. The supernatant was dried *in vacuo* and the dried material redissolved in water saturated with ethyl acetate. This material was chromatographed on a Celite column (1.9 cm diameter x 50 cm length) using solvent A (page 33). The fraction known to contain both i^6Ado and i^6Ade (first 35 ml) was collected, and reduced to a small volume under vacuum. An aliquot of this material was chromatographed on paper in system C. Another aliquot, together with 0.1 mg of unlabelled i^6Ado, was hydrolysed with 1 N HCl (page 35), and subsequently chromatographed on paper in system C. No evidence of radioactive i^6Ado or i^6Ade was found in these experiments (Figure 12). Therefore, it was concluded that 6410 cells do not contain detectable quantities of free i^6Ado or i^6Ade.

It was possible that i^6Ado was present as the 5' monophosphate (i^6AMP). The Celite column was washed with 250 ml of distilled water to elute any i^6AMP. The eluate was dried under vacuum. The material was resuspended in 5 ml of ammonium formate buffer, 0.01 M, pH 9.2, and incubated with 1 mg of chicken intestinal alkaline phosphatase at 37°C for 24 hours. The mixture was rechromatographed on a Celite column (1.9 cm diameter x 50 cm length) using solvent A. No radioactivity occurred in the Celite column fraction known to contain i^6Ado. Therefore, I concluded that 6410 cells do not contain i^6AMP in quantities detectable by this technique.

FIGURE 12

PAPER CHROMATOGRAPHY OF PUTATIVE i^6Ado

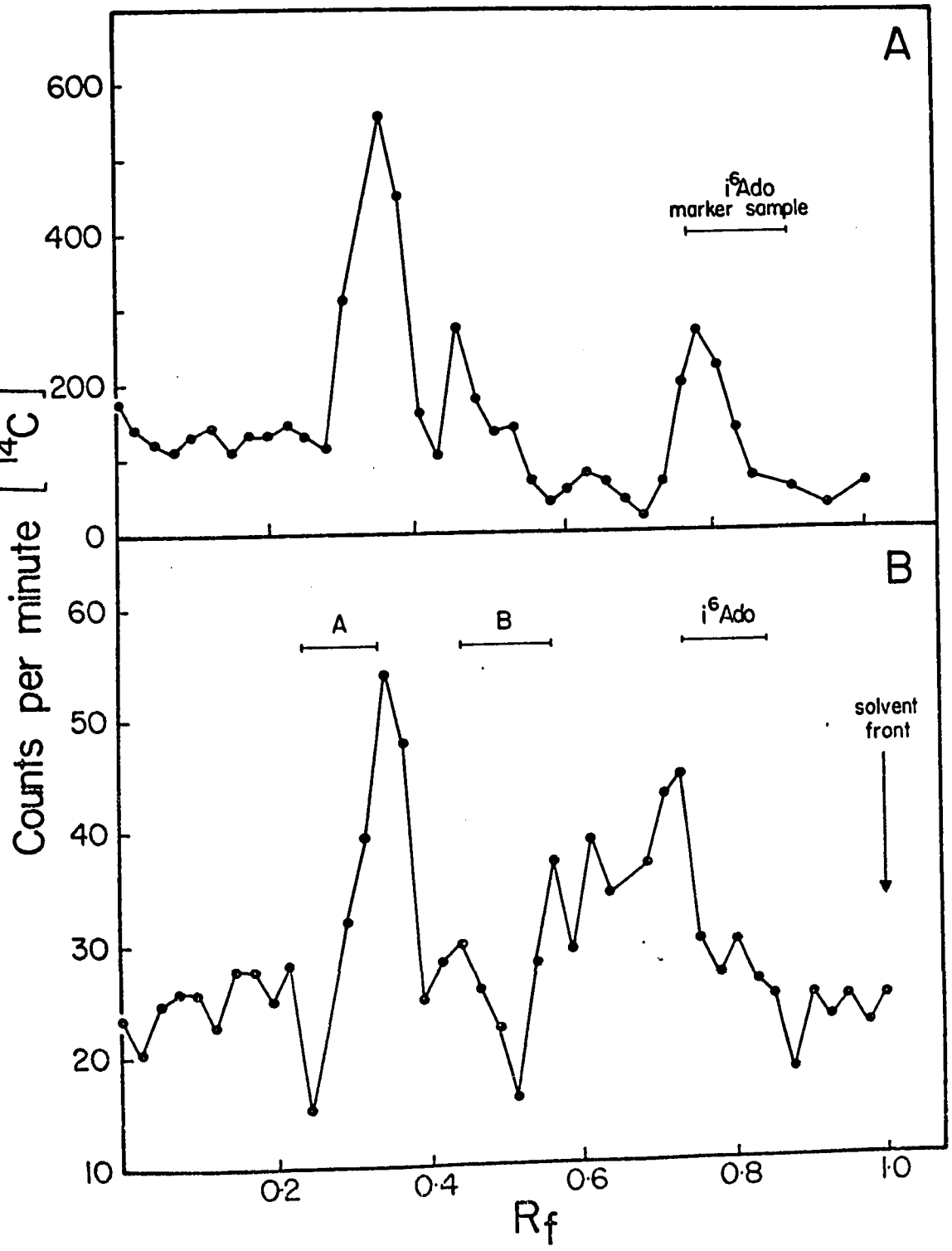
FROM ACID SOLUBLE FRACTION OF 6410 CELLS

Acid soluble material from 6410 cells that had been labelled with [8 -^{14}C] adenosine, together with a sample of i^6Ado (0.2 mg), was chromatographed on a Celite column developed with solvent A, as described in the text.

A. Material from the fraction that contained i^6Ado chromatographed on paper developed in solvent C. The chromatogram was cut into 1 cm strips, and the radioactivity of each strip determined by liquid scintillation counting.

B. Material from the fraction that contained i^6Ado was treated with 1 N HCl as described on page 35. The products were chromatographed on paper developed in solvent C. The chromatogram was cut into 1 cm strips, and the radioactivity of each strip determined by liquid scintillation ccunting.

A,B. Position of acid hydrolysis products of i^6Ado: 3H-7,7-dimethyl-7,8,9-trihydropyrimido-[2,1-i]purine and N^6-(3-hydroxy-3-methylbutyl) adenine.


A
600
400
200
0
i⁶Ado
marker sample
B
60
50
40
30
20
10
A
B
i⁶Ado
solvent
front
Counts per minute [¹⁴C]
0·2
0·4
0·6
0·8
1·0
Rf

The presence of i^6Ado in the growth medium of 6410 cells

Some minor nucleoside components of RNA are released from cells *in vivo* and are found in mammalian urine (Chheda, 1970). Although i^6Ado was not found free in 6410 cells, it was possible that free i^6Ado may have diffused into the culture medium. In that case, if the concentration of i^6Ado inside and outside the cells were of the same order, because the volume of the cells is small compared with that of the medium, then the total quantity of i^6Ado in the medium would be much greater than that in the cells.

Therefore, the medium of a 6410 cell culture (2 litres) that had been labelled with [8 -^{14}C] adenosine for 48 hours during logarithmic growth phase (25 μCi, specific activity 52.6 μCi/μ mole, Schwarz Bioresearch) was extracted four times with ethyl acetate. The extract was chromatographed on a Celite column (1.9 cm diameter x 50 cm length). The first 35 ml of eluent were collected, reduced to a small volume under vacuum and chromatographed on paper in solvent C. The distribution of radioactivity on the chromatogram was not coincident with the i^6Ado standard (Figure 13). Thus it was concluded that i^6Ado was not present in the medium of 6410 cells in quantities detectable by this method. Using a similar technique, i^6Ade was detected in *Corynebacterium fascians* culture medium at concentrations as low as 1.5 nM (pages 135-166).

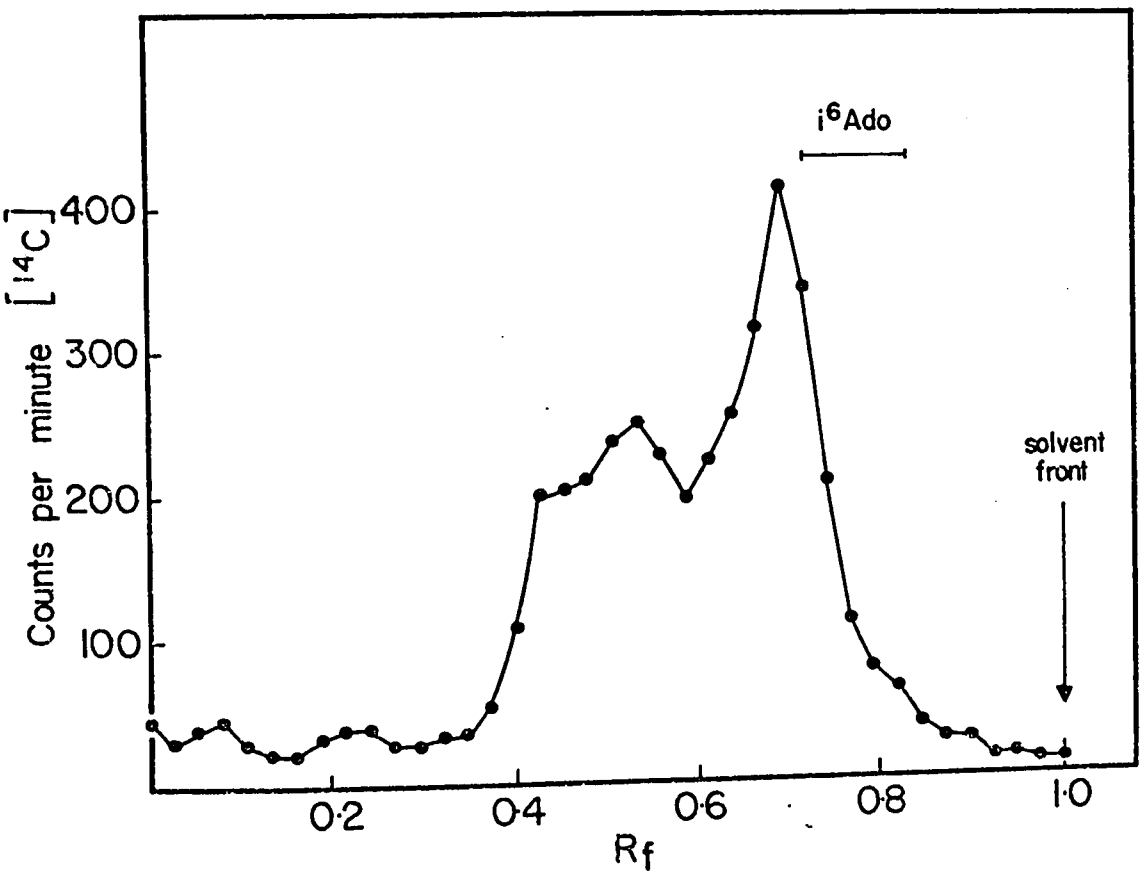
The possibility that i^6AMP rather than i^6Ado or i^6Ade was present in 6410 cell culture medium was investigated. Two litres of medium from a 6410 cell culture that had been labelled with 25 μCi of [8 -^{14}C] adenosine for 48 hours during logarithmic growth phase (specific activity 52.6 μCi/μ mole, Schwarz Bioresearch) was reduced under vacuum to 500 ml.

FIGURE 13

PAPER CHROMATOGRAPHY OF PUTATIVE i^6Ado

FROM 6410 CELL CULTURE MEDIUM

Medium from cultures of 6410 cells that had been labelled with [8 -^{14}C] adenine was extracted with ethyl acetate and chromatographed on a Celite column developed with solvent A as described in the text. Material from the fraction that contained i^6Ado was rechromatographed on paper developed with solvent C. The chromatogram was cut into 1 cm strips and the radioactivity of each strip was determined by liquid scintillation counting.


i⁶Ado
solvent front
Counts per minute [¹⁴C]
400
300
200
100
0·2
0·4
0·6
0·8
1·0
Rf

The concentrated medium was desalted using charcoal as described in the Methods section (page 58). The desalted material was evaporated to dryness, redissolved in 5 ml of ammonium formate buffer, 0.01 M, pH 9.2, and 1 mg of chicken intestinal alkaline phosphatase was added. The mixture was incubated at 37°C for 24 hours, and then chromatographed on a Celite column (1.9 cm diameter x 50 cm length) using solvent A. The fraction known to contain i^6Ado was not radioactive. Therefore, I concluded that the culture medium of 6410 cells does not contain i^6AMP in quantities that are detectable by this technique.

The biosynthesis of i^6Ado in the tRNA of 6410 cells

Because 6410 cells were killed by i^6Ado, a normal constituent of their tRNA, the metabolism and biosynthesis of i^6Ado in the tRNA of 6410 cells was investigated, first *in vivo*, then *in vitro*.

Studies of i^6Ado biosynthesis in cultures of 6410 cells

It had been shown that mevalonic acid (MVA) added to the growth medium of *Lactobacillus acidophilus* is incorporated into the bacteria. Mevalonic acid is a precursor of the Δ^2-isopentenyl sidechain of the i^6Ado moiety of the tRNA (Fittler *et al.*, 1968a;Peterkofsky, 1968). Therefore, attempts were made to label the isopentenyl sidechain of i^6Ado moieties of 6410 cell tRNA by adding [2 -^{14}C] MVA to the growth medium of these cells. [2 -^{14}C] MVA (New England Nuclear Corporation, specific activity 2.3 μCi/μ mole), [2 -^{14}C] mevalonic acid lactone, (specific activity 2.9 μCi/μ mole, New England Nuclear Corporation), and a methyl ester of [2 -^{14}C] MVA were each used. The MVA methyl ester was synthesized by Dr. Michael Brown, from [2 -^{14}C] MVA-L-lactone (New England Nuclear Corporation) and had the structure:

$$(HO)CH_2-CH_3=C-CH_3(OH)-CH_2-CH_3$$

and a specific activity of 2.9 μCi/μ mole.

In each experiment a culture of 6410 cells (4 litres) was grown to early logarithmic growth phase. Either 100 μCi of [2 -^{14}C] MVA, 50 μCi of [2 -^{14}C] MVA lactone, or 50 μCi of [2 -^{14}C] MVA methyl ester was added to the culture, which was grown for a further 72 hours. The cells were harvested by centrifugation at 3,000 g for 10 minutes at 0°C, and the RNA extracted (page 35).

The quantity of RNA was determined by recording the absorbance at 260 nm and 280 nm of an aliquot of the material. Another aliquot of the RNA was dissolved in 1 ml of water and placed in 15 ml of dioxane cocktail I (page 41) and its radioactivity determined by liquid scintillation counting. In no case was the radioactivity of the RNA above background levels, indicating that MVA, MVA lactone, and MVA methyl ester were not incorporated into 6410 cell RNA.

Therefore, it was concluded that MVA, MVA lactone, or the methyl ester of MVA could not be used as specific precursors to study the biosynthesis of the Δ^2-isopentenyl sidechain of i^{6}Ado in the 6410 cell tRNA *in vivo*.

In vitro studies of i^{6}Ado biosynthesis

Kline *et al*. (1969) have shown that yeast and mammalian cells contain an enzyme that catalyzes the transfer of Δ^2-isopentenyl groups from Δ^2-isopentenyl pyrophosphate to preformed tRNA. The purpose of the following investigation was to determine whether 6410 cells contain this enzyme activity.

When E. coli tRNA was incubated with an enzyme system from 6410 cells (page 57) and [2 -^{14}C] MVA, ^{14}C was incorporated into the tRNA. Under the conditions of assay, the specific activity of the tRNA was between 400 cpm and 1200 cpm per 25 A_{260} units. The identity of the radioactive material in the tRNA was determined in three ways:

(i) A 1 ml aliquot of the radioactive tRNA was mixed with unlabelled i^{6}Ado (0.25 mg) and hydrolysed in 1 N HCl at 100°C for 15 minutes, with unlabelled i^{6}Ado (0.25 mg). The mixture was chromatographed on Whatman 3 mm paper in solvent C. The chromatogram was cut into strips and its radioactivity determined by liquid scintillation counting (Figure 14). The tRNA was not fully hydrolysed and some radioactivity remained at the origin of the chromatogram. The other radioactive areas corresponded to the two products of acid hydrolysis of the i^{6}Ado marker (page 35).

(ii) An aliquot (2 ml) of the tRNA solution was dried in a rotary evaporator and hydrolysed with 0.3 N KOH (page 39) to form the constituent ribonucleotides, treated with alkaline phosphatase to form the corresponding nucleosides (page 39), and chromatographed on a 1.9 cm diameter x 50 cm length Celite column (page 32), using solvent A. The first 35 ml fraction that contains i^{6}Ado was reduced to a small volume on a rotary evaporator, and an aliquot was chromatographed on Whatman I paper using solvent C. All the radioactivity was located with the i^{6}Ado standard (Figure 15A).

(iii) Another aliquot of the first 35 ml fraction from the Celite column described above was hydrolysed by 1 N HCl (page 35) and also chromatographed on Whatman I paper in solvent C. The radioactivity

FIGURE 14

PAPER CHROMATOGRAM OF AN ACID HYDROLYSATE OF E. COLI tRNA THAT WAS A SUBSTRATE FOR Δ^2-ISOPENTENYL PYROPHOSPHATE: tRNA Δ^2-ISOPENTENYL TRANSFERASE FROM 6410 CELLS

One hundred A_{260} units of E. coli tRNA was used as a substrate for the transferase enzyme from 6410 cells. The assay was done using [2 -^{14}C] mevalonic acid as described in the text. An aliquot of the tRNA recovered (12 A_{260} units) was added to 0.2 mg of unlabelled i^6Ado and the mixtures hydrolysed with 1N HCl for 15 minutes at 100°C. The product was chromatographed on paper using solvent C. The chromatogram was cut into 1.25 cm strips and the radioactivity of each strip was determined by liquid scintillation counting.

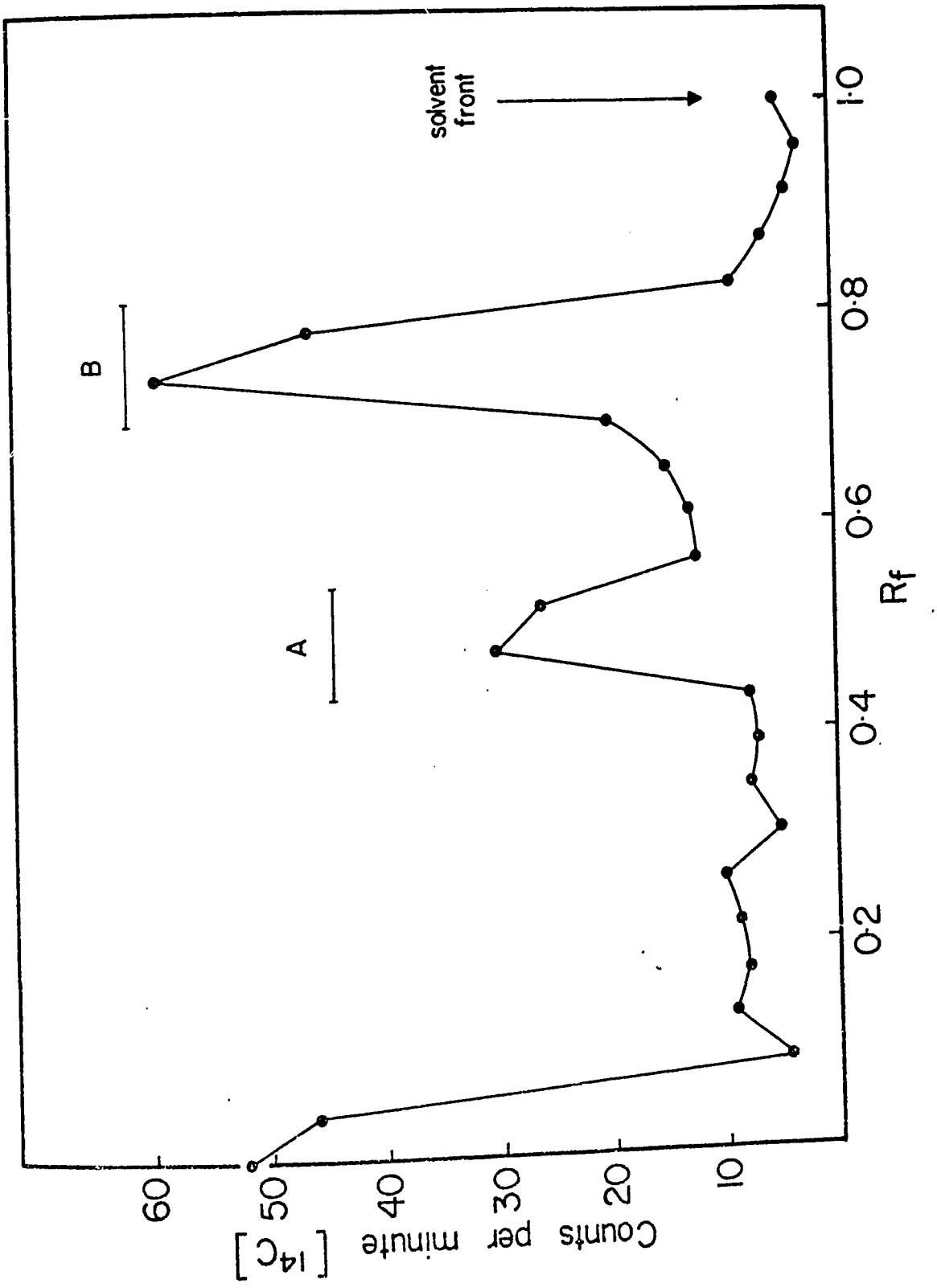

Counts per minute [14C]
60
50
40
30
20
10
0·2
0·4
0·6
0·8
1·0
Rf
A
B
solvent front

FIGURE 15

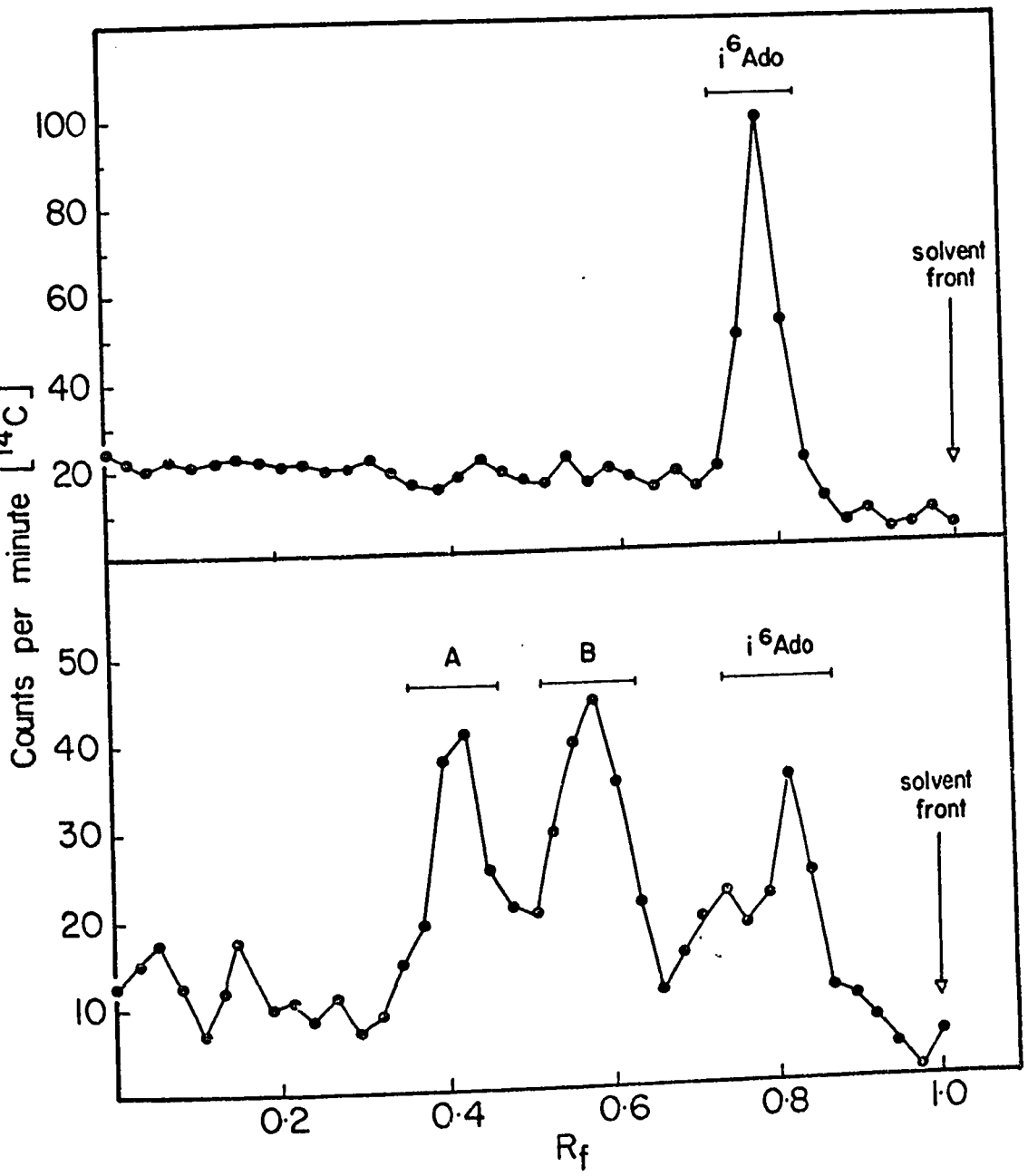
A. PAPER CHROMATOGRAM OF PUTATIVE i^6Ado FROM E. COLI tRNA THAT WAS A SUBSTRATE FOR Δ^2-ISOPENTENYL PYROPHOSPHATE: tRNA Δ^2-ISOPENTENYL PYROPHOSPHATE TRANSFERASE FROM 6410 CELLS

One hundred A_{260} units of E. coli tRNA was used as a substrate for the transferase enzyme from 6410 cells. The assay was done using [2 -^{14}C] mevalonic acid as described in the text. An aliquot of the tRNA recovered (24 A_{260} units) was hydrolysed with 0.3 M KOH as described on page 39. The resulting nucleotides were converted to the respective nucleosides by treatment with alkaline phosphatase as described on page 39. The mixture, together with 0.2 mg of unlabelled i^6Ado, was chromatographed on a 2.5 cm diameter x 50 cm height Celite column developed with solvent C. Half of the material that chromatographed with i^6Ado on the Celite column was rechromatographed on paper developed with solvent C. The chromatogram was cut into 1 cm strips and the radioactivity of each strip determined by liquid scintillation counting.

B. PAPER CHROMATOGRAM OF ACID HYDROLYSATE OF PUTATIVE i^6Ado FROM E. COLI tRNA THAT WAS A SUBSTRATE FOR Δ^2-ISOPENTENYL PYROPHOSPHATE: tRNA Δ^2-ISOPENTENYL PYROPHOSPHATE TRANSFERASE FROM 6410 CELLS

One half of the material that chromatographed in the same fraction from the Celite column described in A. above was hydrolysed with 1 N HCl for 15 minutes at 100°C. The hydrolysate was rechromatographed on paper and developed with solvent C. The chromatogram was cut into 1 cm strips and the radioactivity of each strip determined by liquid scintillation counting.

A,B. Position of acid hydrolysis products of i^6Ado: 3H-7,7-dimethyl-7,8,9-trihydropyrimido-[2,1-i]purine and N^6-(3-hydroxy-3-methylbutyl) adenine.


i⁶Ado
solvent front
100
80
60
40
20
A
B
i⁶Ado
solvent front
50
40
30
20
10
Counts per minute [¹⁴C]
0·2
0·4
0·6
0·8
1·0
Rf

was located with the i^6Ado standard and with the two characteristic products of acid hydrolysis of i^6Ado (Figure 15B).

This indicates that 6410 cells contain an enzyme system capable of isopentenylating an adenine residue in E. coli tRNA to form i^6Ado, using [2 -^{14}C] MVA as a precursor. This is analogous to an enzyme pathway that is found in other organisms such as Lactobacillus acidophilus, (Fittler et al., 1968a; Peterkofsky, 1968), yeast (Kline et al., 1969), tobacco pith tissue (Chen and Hall, 1969), and E. coli (Bartz et al., 1970).

A comparison of tRNA from various sources as a substrate for Δ^2-isopentenyl pyrophosphate: tRNA Δ^2-isopentenyl transferase from 6410 cells

It was thought possible that when 6410 cells were treated with exogenous i^6Ado the synthesis of i^6Ado in the tRNA might be reduced, perhaps by a feedback mechanism. Thus the ability of Δ^2-isopentenyl pyrophosphate: tRNA Δ^2-isopentenyl transferase from 6410 cells to catalyze the transfer of Δ^2-isopentenyl groups onto tRNA molecules from 6410 cells treated with i^6Ado was investigated.

tRNA was prepared from 6410 cells grown to late logarithmic phase. Other 6410 cells were grown in the presence of 2.2 μM i^6Ado from the beginning of logarithmic phase. This was the maximum concentration of i^6Ado that did not affect the viability of 6410 cell cultures. After 72 hours the cells were harvested by centrifugation for 10 minutes at 3,000 g at 0°C. tRNA was prepared from the cells by the method described on page 38.

The enzyme preparation from 6410 cells was found to have Δ^2-isopentenyl pyrophosphate: tRNA Δ^2-isopentenyl transferase activity when assayed with E. coli tRNA as a substrate. No activity was demonstrated when 6410 cell tRNA or tRNA from 6410 cells that had been treated with i^6Ado were used as substrates. These results indicate that Δ^2-isopentenyl pyrophosphate: tRNA Δ^2-isopentenyl transferase from 6410 cells does not have the ability to hyper-isopentenylate 6410 cell tRNA. Also, cells grown in the presence of i^6Ado at the maximum concentration which does not cause the cells to die do not have isopentenyl-deficient tRNA molecules. The results of a typical experiment are shown in Table 4. I conclude that under the conditions of the experiment there is no evidence for the existance of a feedback mechanism whereby i^6Ado in the growth medium of 6410 cells inhibits the transfer of Δ^2-isopentenyl groups to the tRNA molecules of the cells.

The degradation of i^6Ado by 6410 cells

Hanoune and Agarwal (1970) have estimated the half-life of tRNA in rat liver to be five days. A number of the characteristic components of tRNA have been found in human urine (Chheda, 1970). These observations indicate that there is some degradation of tRNA in vivo. It might be expected, therefore, that i^6Ado, together with other modified nucleosides, would be released from tRNA as a result of degradation. However, no free i^6Ado has been reported to be isolated from animal sources. This finding, together with the observation that i^6Ado is toxic to 6410 cells, implies that 6410 cells might possess a mechanism by which the i^6Ado that is released by tRNA degradation could be rendered less toxic. Some evidence for such a mechanism has already been reported by Hall et al.

TABLE 4

Δ^2-ISOPENTENYL PYROPHOSPHATE: tRNA Δ^2-ISOPENTENYL TRANSFERASE ACTIVITY IN 6410 CELLS

Enzyme Source	tRNA Substrate Source	cpm/25 A_{260} Units of tRNA
6410 cells	E. coli	1,115
6410 cells	6410 cells	52
6410 cells	6410 cells grown in the presence of 2.25 μM i^6Ado for 72 hours	0

Three g of 6410 cells were homogenized in a French Press in 15 ml of a buffer; 50 mM Tris-Cl, pH 7.5, 20 mM mercaptoethanol, 5 mM magnesium chloride. The homogenate was centrifuged at 40,000 g for 30 minutes at 4°C. Four ml aliquots of the supernatant were added to a solution of ATP (50 mg), 2.15×10^6 cpm of [2 -^{14}C] mevalonic acid, (specific activity 2.95 μCi/μ mole), and 100 A_{260} units of tRNA, at pH 7.5, and incubated for 1 hour at 30°C. tRNA was isolated from the reaction mixture by the method of Kline et al. (1969) and the radioactivity of an aliquot determined by liquid scintillation counting.

(1971) who showed that human and other bone marrow contains strong adenosine aminohydrolase activity that catalyzes the conversion of i^6Ado to inosine. Therefore, I investigated whether 6410 cells contained an enzyme system capable of degrading i^6Ado.

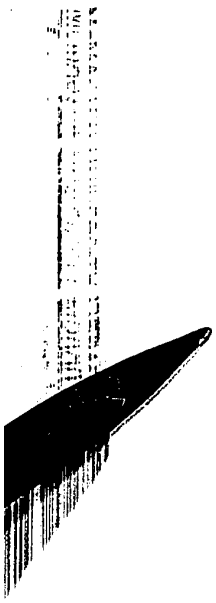
Freshly prepared, purified [8 -^{14}C] i^6Ado (specific activity 5 μCi/μ mole) was synthesized by Dr. D. Whitty using the method of Paces et al. (1971). RPMI 6410 cells were harvested by centrifugation for 10 minutes at 10,000 g at 4°C and resuspended in twice their volume of a buffer containing 50 mM tris HCl, 5 mM magnesium chloride, 20 mM 2-mercaptoethanol, pH 7.5. The cells were disrupted by ultrasonic vibration for two periods of 10 seconds, during which the mixture was cooled in an ice-bath. The homogenate was centrifuged for 30 minutes at 20,000 g at 4°C and 25 μl of the supernatant was added to 10,000 dpm of [8 -^{14}C] i^6Ado in 2 ml of a buffer containing 50 mM sodium phosphate, 0.4 mM magnesium chloride, pH 6.5. As a control a portion of the cell homogenate was heated to 100°C in a waterbath for 2 minutes, after which an aliquot of 25 μl was added to 2 ml of the phosphate buffer and 10,000 dpm of [8 -^{14}C] i^6Ado. The reaction vessels were sealed and incubated at 37°C in a waterbath. Samples (200 μl) were removed at various time intervals. Unlabelled i^6Ado (0.1 mg) was added to each aliquot before it was chromatographed on paper using solvent B. Areas of ultraviolet absorption were defined on the chromatograms and radioactivity was determined using a radiochromatogram scanner (Figure 16). N^6-(Δ^2-Isopentenyl)adenosine was converted to a single product that had an Rf of 0.5 when chromatographed on paper using 1 M ammonium borate pH 9.0: 95% ethanol (9:1 v/v) (solvent B).

FIGURE 16

THE IN VITRO DEGRADATION OF [8 -^{14}C] i^{6}Ado

BY AN ENZYME FROM 6410 CELLS

For each assay 1 n mole of [8 -^{14}C] i^{6}Ado (specific activity 5 μCi/μ mole) was dissolved in 2 ml of a buffer that contained 50 mM NaH_2PO_4, 0.4 mM $MgCl_2$, pH 6.5 was incubated with 25 μl of an homogenate of 6410 cells prepared as described in the text. Control assays were done using 25 μl of an homogenate of 6410 cells that had been heated to 100°C for 1 minute. At the intervals indicated 200 μl aliquots of the assays were chromatographed on paper in solvent B. The distribution of radioactivity was determined using a radiochromatogram scanner.



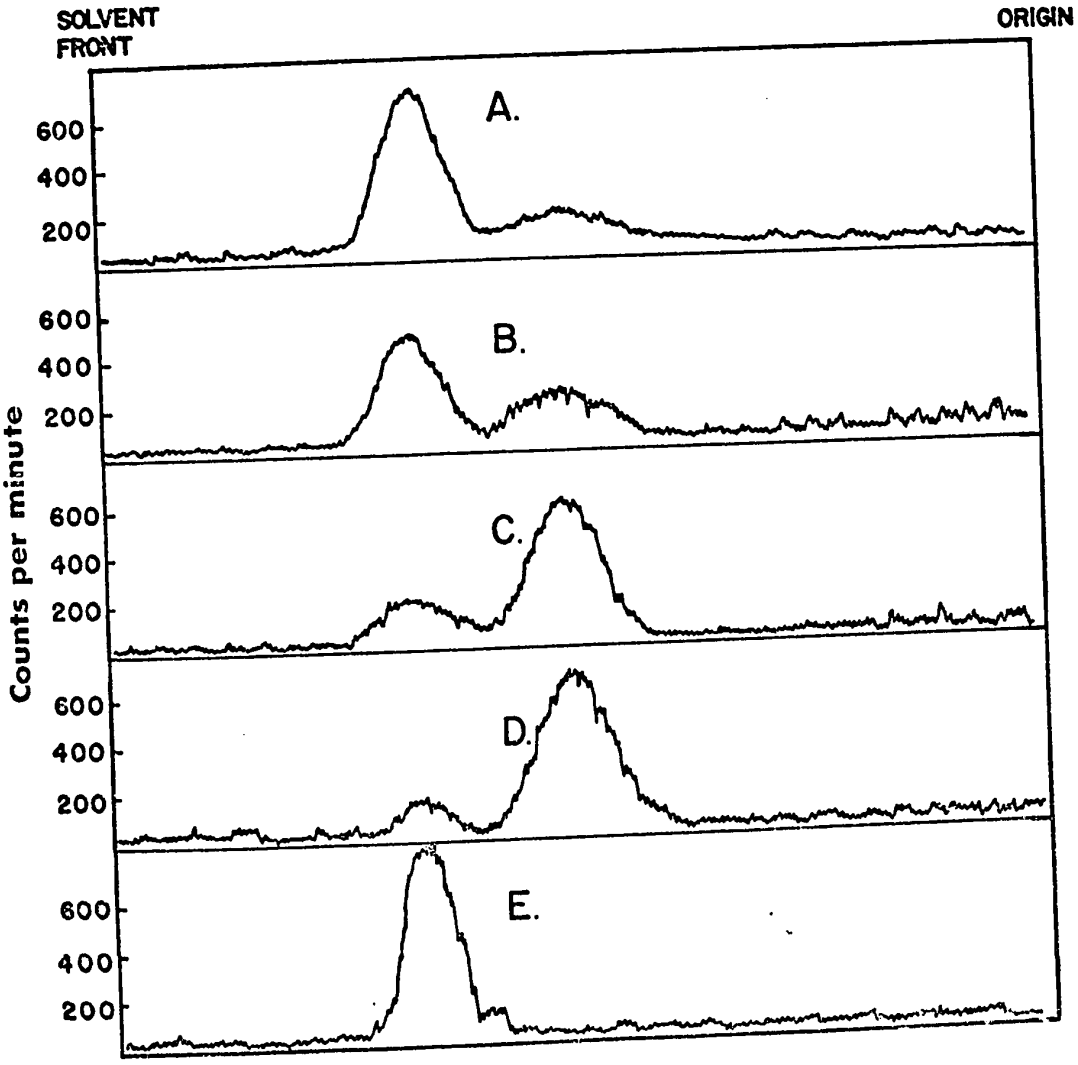
A. 1 hour incubation.

B. 3 hours incubation.

C. 16 hours incubation.

D. 24 hours incubation.

E. 24 hours incubation - heated enzyme control.


SOLVENT FRONT
ORIGIN
A.
B.
C.
D.
E.
600
400
200
Counts per minute

The radioactive product was rechromatographed on paper using solvent B with unlabelled samples of theoretical metabolic products of i^6Ado. These included adenine, adenosine, $\underline{N}^6$-(4-hydroxy-3-methylbut-2-enyl)adenosine, $\underline{N}^6$-(4-hydroxy-3-methylbut-2-enyl)adenine, $\underline{N}^6$-(3-hydroxy-3-methylbutyl)adenosine, $\underline{N}^6$-(3-hydroxy-3-methylbutyl)adenine, inosine, and hypoxanthine. The product cochromatographed with i^6Ade but not with any of the other compounds (Figure 17).

The product also chromatographed coincidentally with i^6Ade on paper using solvent C (Figure 18A).

A sample of i^6Ade (0.2 mg) was mixed with an aliquot of the radioactive product (400 µl). The mixture was dried _in vacuo_, redissolved in 1 M HCl (1 ml) and incubated at 100°C for 15 minutes. The product was chromatographed on paper using solvent C. After hydrolysis all the radioactivity chromatographed coincidentally with the characteristic products of acid hydrolysis of i^6Ade (page 35). These data are shown in Figure 18B.

From these experiments I concluded that 6410 cells contain an enzyme activity that metabolizes i^6Ado to i^6Ade. No other metabolic products were detected.

Human, avian and rabbit bone marrow contain an adenosine deaminase activity that converts i^6Ado to inosine (Hall and Mintsioulis, in press; Hall _et al._, 1971). Homogenates of 6410 cells were assayed for this activity using a spectrophotometric assay.

Inosine has a different absorption maximum in the ultraviolet spectrum from adenosine. The rate of decrease in light absorption at 265 nm is proportional to the rate of deamination of the adenosine.

FIGURE 17

PAPER CHROMATOGRAPHY OF THE ENZYMATIC DEGRADATION PRODUCT OF i^6Ado

An aliquot (100 µl) of the radioactive product that was formed from [8 -^{14}C] i^6Ado upon incubation with an homogenate of 6410 cells was chromatographed on paper with solvent C, together with some possible metabolic products of i^6Ado.

A. The position of the unlabelled compounds:

a. Adenine

b. Adenosine

c. N^6-(4-hydroxy-3-methylbut-2-enyl)adenine

d. N^6-(4-hydroxy-3-methylbut-2-enyl)adenosine

e. i^6Ado

f. i^6Ade

g. Hypoxanthine

h. Inosine

i. N^6-(3-hydroxy-3-methylbutyl)adenosine

j. N^6-(3-hydroxy-3-methylbutyl)adenine

B. The distribution of radioactivity on the chromatogram determined using a radiochromatogram scanner.

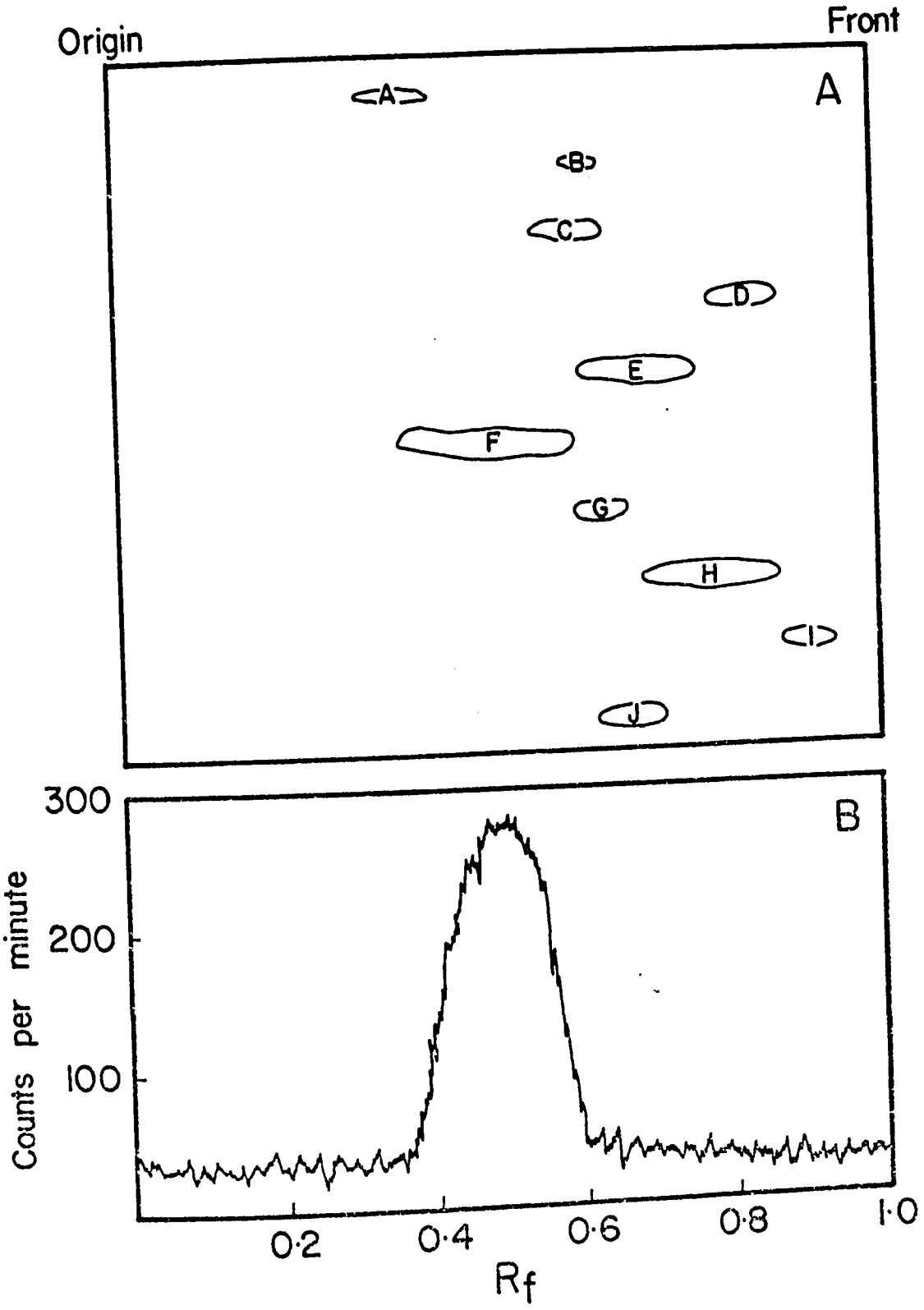

Origin
Front
A
A
B
C
D
E
F
G
H
I
J
B
300
200
100
Counts per minute
0·2
0·4
0·6
0·8
1·0
Rf

FIGURE 18

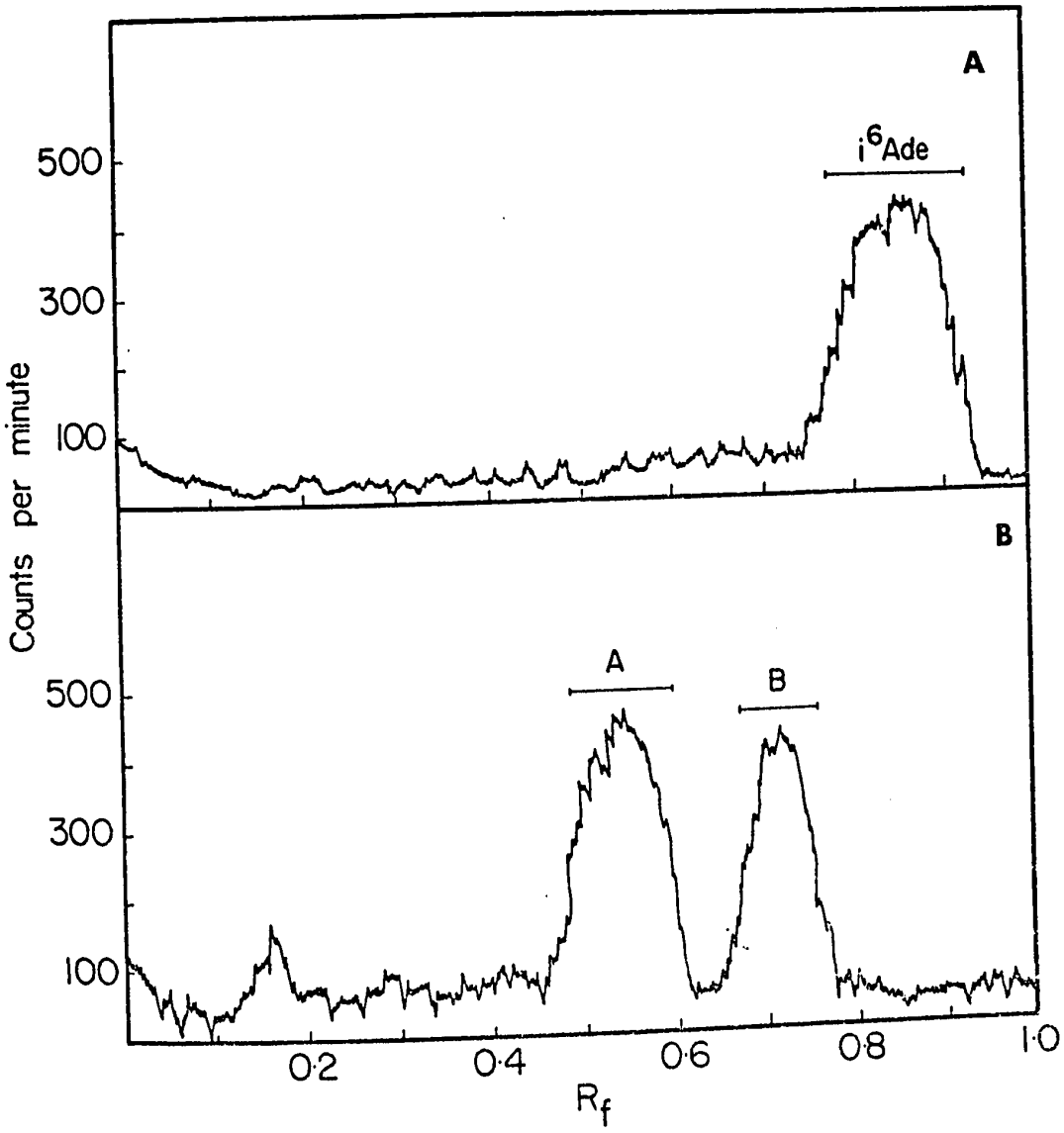
A. PAPER CHROMATOGRAM OF PUTATIVE i^6Ade

An aliquot of (100 µl) of the radioactive product that was formed from [8 -^{14}C] i^6Ado upon incubation with an homogenate of 6410 cells was chromatographed on paper in solvent C. The distribution of radioactivity on the chromatograms was determined using a radiochromatogram scanner.

B. PAPER CHROMATOGRAM OF THE PRODUCTS OF ACID HYDROLYSIS OF PUTATIVE i^6Ade

Unlabelled i^6Ade (0.2 mg) was added to 200 µl of the radioactive product that was formed from [8 -^{14}C] i^6Ado. The mixture was dried *in vacuo* and incubated with 1 N HCl for 15 minutes at 100°C. The mixture was chromatographed on paper with solvent C. The distribution of radioactivity was determined using a radiochromatogram scanner.

A,B. Position of acid hydrolysis products of i^6Ado: 3H-7,7-dimethyl-7,8,9-trihydropyrimido-[2,1-i]purine and N^6-(3-hydroxy-3-methylbutyl) adenine.


A
i6Ade
500
300
100
B
A
B
500
300
100
Counts per minute
0·2
0·4
0·6
0·8
1·0
Rf

If, in a similar reaction, the isopentenyl sidechain is removed from i^6Ado, a similar decrease occurs. This is best observed at 268 nm.

The change in optical density of the solution at 265 nm in the case of adenosine, or at 268 nm in the case of i^6Ado was observed by placing the reaction mixtures in quartz cuvettes maintained at 37°C by a heating block in a Gilford 2000 recording spectrophotometer.

The cell homogenate was prepared as described for the preceding experiments in which radioactive i^6Ado was used as a substrate. An aliquot of the cell homogenate (0.1 ml) was diluted by the addition of 9 volumes of a buffer containing 50 mM sodium phosphate, 0.4 mM magnesium chloride, pH 6.5. Twenty five µl of this solution was added to 3 ml of the same buffer to which 90 µM adenosine had been added and had been prewarmed to 37°C in an optical quartz cuvette in a spectrophotometer. The results of a typical assay are shown in Figure 19, and indicate that 6410 cells contain adenosine deaminase.

A similar assay system was used to determine whether i^6Ado were converted to inosine by an adenosine deaminase activity from these cells. In these experiments adenosine was replaced in the reaction mixture by i^6Ado at concentrations of 30 µM, 60 µM and 90 µM. The absorption of the mixture was measured at 268 nm. No change in optical density was observed (Figure 18 B,C,D). This indicates that the enzyme preparation from 6410 cells did not remove the isopentenyl sidechain from i^6Ade under the conditions of the experiment.

It can be concluded from these studies that 6410 cells contain an enzyme activity that converts i^6Ado to i^6Ade. Unlike bone marrow (Hall et al., 1971) 6410 cells do not contain an adenosine aminohydrolase activity that is capable of converting i^6Ado to inosine.

FIGURE 19

ADENOSINE AMINOHYDROLASE ACTIVITY FROM 6410 CELLS

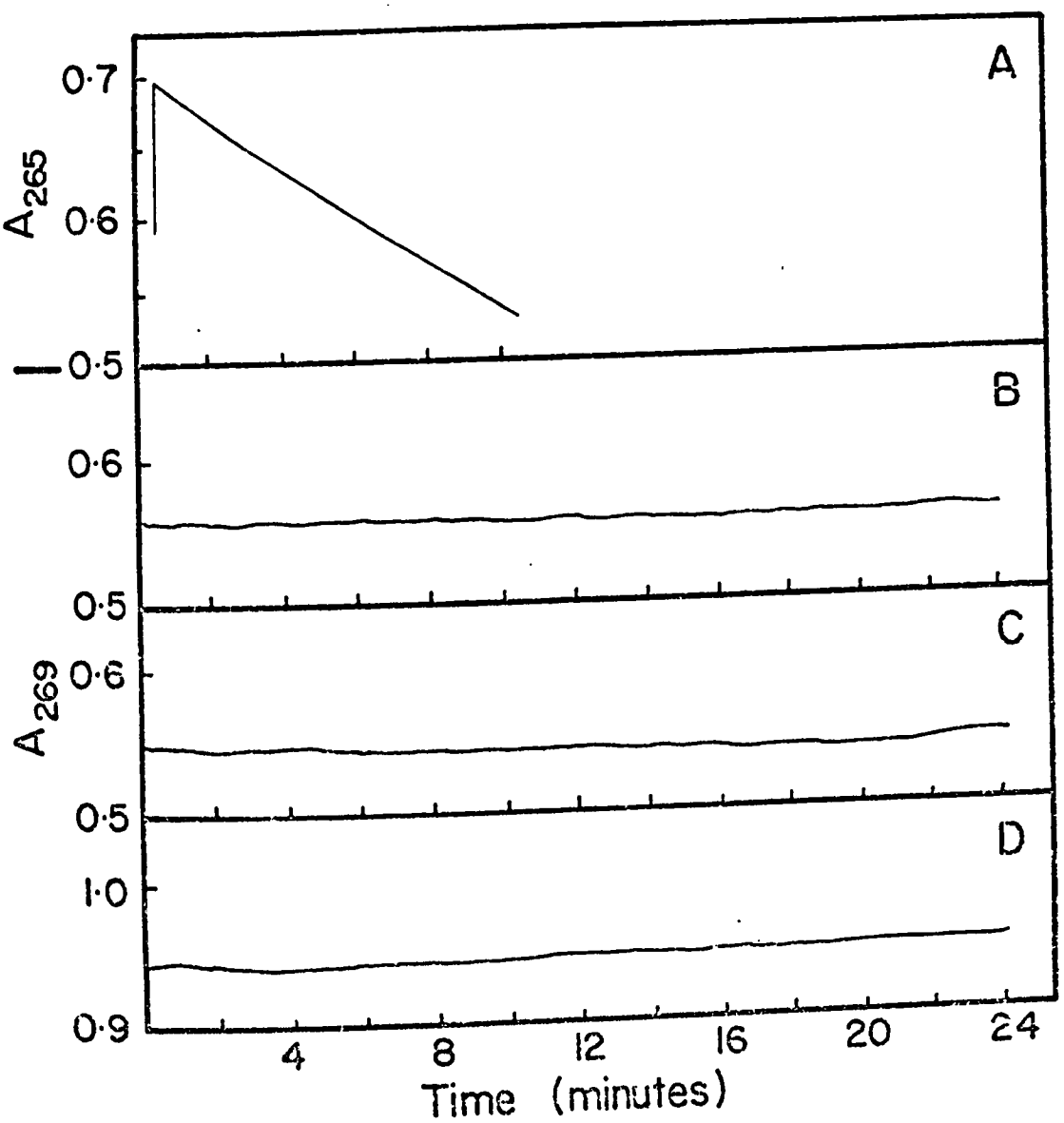
An homogenate was prepared from 6410 cells as described in the text (page 100). In each assay 25 µl of the homogenate was added to 3 ml of a buffer: 50 mM NaH_2PO_4, 0.4 mM $MgCl_2$ pH 6.5, that contained adenosine (90 µM) or i^6Ado (30 µM, 60 µM or 90 µM). The mixture was incubated in optical quartz cuvettes of 1 cm path length in a spectrophotometer. The temperature of the cuvette housing was maintained at 37°C by a thermostatically controlled heating jacket. The rate at which the absorption of light decreased was observed at 265 nm or at 268 nm.

A. 90 µM adenosine

B. 30 µM i^6Ado

C. 60 µM i^6Ado

D. 90 µM i^6Ado


A
B
C
D
0·7
0·6
0·5
A_{265}
0·6
0·5
0·6
0·5
A_{269}
1·0
0·9
4
8
12
16
20
24
Time (minutes)

Differentiation of tobacco pith tissue induced by i^6Ado

The growth and morphological changes induced in KX-4 tissue by i^6Ado

The concentrations of i^6Ado required to induce differentiation in KX-4 tissue were ascertained. This enabled a comparison to be made between the responses to i^6Ado of normal KX-4 tissue and a variant strain used in later experiments.

Callus tissue from stock cultures of KX-4 tobacco pith, that had been subcultured 35 days previously, was divided freehand into small pieces. Each piece weighed between 20 mg and 30 mg. Groups of 3 pieces of tissue were weighed under sterile conditions and planted in conical flasks containing 100 ml of X medium or X medium containing i^6Ado. The final concentrations of i^6Ado in the medium were 0.015 μM, 0.03 μM, 0.15 μM, 0.3 μM, 0.6 μM, 1.5 μM, 3 μM and 6 μM. The tissue was grown for 42 days in the dark at room temperature and then weighed. The pieces of callus were dried at 55°C for 24 hours and reweighed to determine their dry weight (Chen, 1968, personal communication). Pieces of tissue grown in the presence of i^6Ado showed greater increases in both fresh weight and dry weight than control tissues grown on X medium that contained no added i^6Ado (Figure 20). The greatest increase in both fresh weight and dry weight of tissue occurred in cultures treated with 0.6 μM i^6Ado. At high concentrations of i^6Ado (3 μM and 6 μM) the fresh and dry weights of the tissue were less than those of the control tissue. However, tissue treated with high concentrations of i^6Ado had a greater dry weight to fresh weight ratio than did the control tissues, or tissue treated with lower concentrations of i^6Ado. These results indicate that although high concentra-

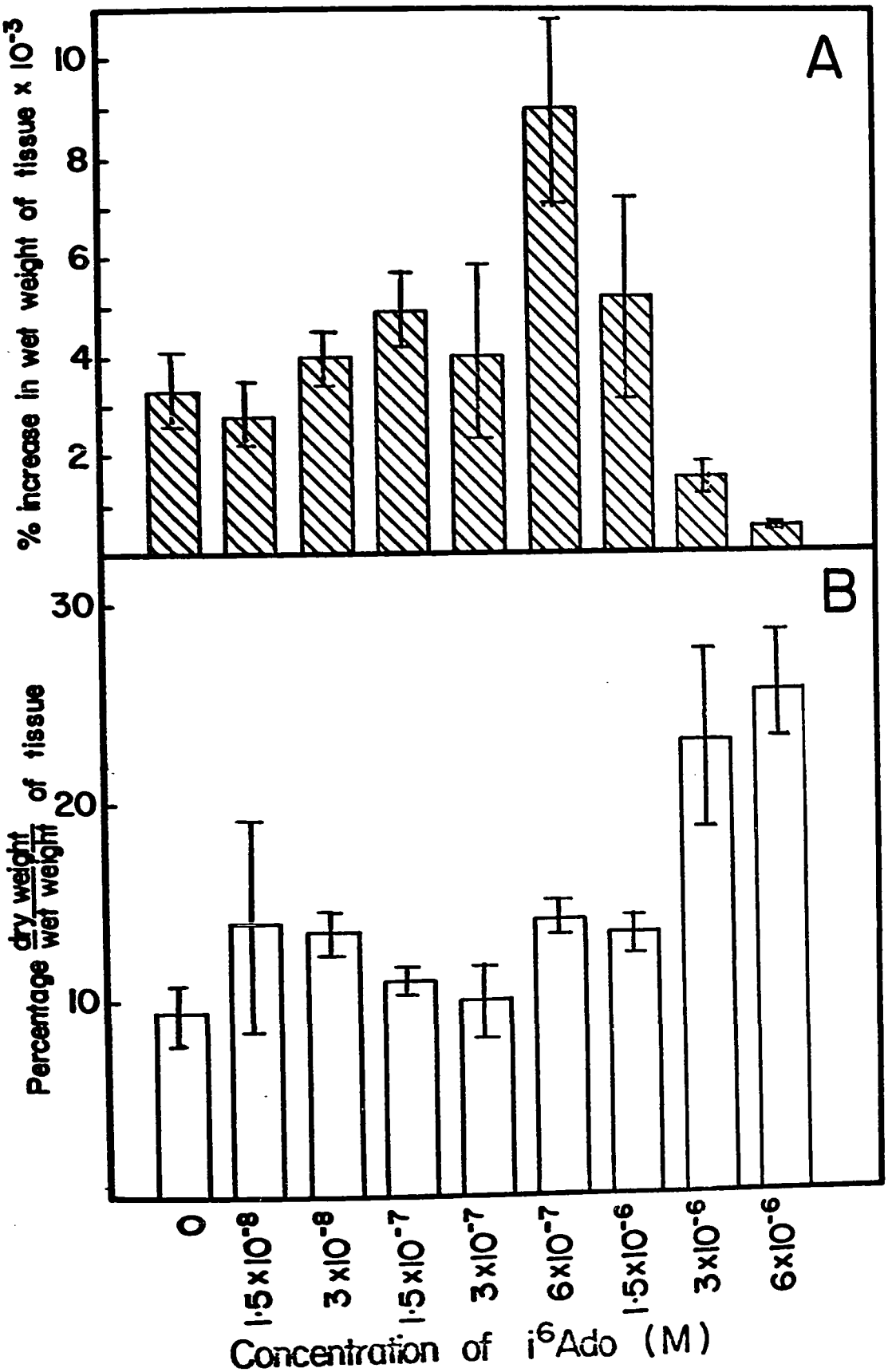
FIGURE 20

STIMULATION OF GROWTH OF KX-4 TOBACCO PITH TISSUE BY i^6Ado

KX-4 callus tissue from stock cultures that had been sub-cultured 35 days previously was divided freehand into pieces that weighed between 20 mg and 30 mg. Three pieces of tissue were placed into each of four 250 ml conical flasks that contained 100 ml of X medium, or into each of four flasks that contained X medium and a particular concentration of i^6Ado from 0.015 μM to 6 μM. The tissue was incubated in the dark at room temperature for 42 days. The tissue in each flask was weighed to determine the fresh weight of the callus, dried at 55°C for 24 hours and reweighed to determine the dry weight.

A. The mean increase in fresh weight of the callus ± the standard error of the mean expressed as a percentage of the initial weight of the tissue.

B. Represents the mean, ± the standard error of the mean values of the dry to fresh weight ratio of the callus expressed as a percentage of the fresh weight.


A
B
% increase in wet weight of tissue x 10⁻³
Percentage dry weight / wet weight of tissue
10
8
6
4
2
30
20
10
0
1·5x10⁻⁸
3x10⁻⁸
1·5x10⁻⁷
3x10⁻⁷
6x10⁻⁷
1·5x10⁻⁶
3x10⁻⁶
6x10⁻⁶
Concentration of i⁶Ado (M)

tions of i^6Ado inhibit the increase in both the fresh and dry weights of pith tissue, the inhibition of the increase in dry weight is less than of fresh weight.

The concentration of i^6Ado in the culture medium also had a profound effect on the morphology of the pith tissue. Callus grown on medium containing concentrations of i^6Ado up to 0.6 μM, the concentration that induced maximum growth, had a friable appearance, was amber coloured, and showed no evidence of differentiation. Tissue grown in the presence of higher concentrations of i^6Ado was more compact and had a patchy, white pigmentation. At high i^6Ado concentrations, (3 μM or higher) the tissue had the consistency of unripe pears, intense white pigmentation and showed evidence of differentiation. Buds formed on the surface of the callus. When allowed to grow for periods of about 90 days these buds developed with shoot and leaf formation.

Because the objective of these experiments was to define some of the initial events leading to differentiation of this tissue, subsequent experiments were done using concentrations of i^6Ado greater than 3 μM. In the growth experiments described above, these concentrations were shown to be sufficient to induce differentiation in KX-4 tissue.

A KX-4 tissue variant which had lost the ability to differentiate

During routine maintenance of KX-4 tissue in our laboratory I observed that a spontaneous variation had occurred in one callus. The variant tissue (KX-4/R1) differed from the normal tissue in its response to i^6Ado. The growth of the tissue did not appear to be inhibited by high concentrations of cytokinin as was observed in the normal KX-4

tissue (page 111) but did require cytokinins for growth. Upon treatment with high concentrations of i^6Ado (9 μM) the tissue initially appeared to be differentiating, but buds and shoots never formed. The appearance of this strain of tissue afforded an opportunity to study some of the events that occur in pith tissue following the entry of cytokinin into the cells, before the formation of organs. It also provided a system to investigate further the observations of Thorpe and Murashige (1968, 1970) that, in tobacco pith tissue that forms organs, a rise and subsequent fall in the starch content of the tissue occurs, whereas in tissues that do not form organs no such accumulation of starch occurs.

Cellular events which occur in tobacco pith tissue KX-4/R1 following administration of i^6Ado

Pieces of KX-4/R1 callus that had grown for 35 days since their last subculture were divided freehand into pieces each weighing about 20 mg. The pieces were cultured on X medium in the absence of any added cytokinin. During this period the tissue became friable and loose. After 10 days the pieces of callus were transferred to 250 ml conical flasks each containing 100 ml of X medium and 9 μM i^6Ado. At this time, and at 2 to 3 day intervals thereafter, samples of tissue were removed and prepared for histological examination by the methods described on pages 48 to 51.

(a) General features

The tissue was grown in the absence of cytokinin for 10 days, then transferred to a medium containing 9 μM i^6Ado. Three samples of the tissue were immediately removed for histological examination. At this time the tissue had several distinctive features.

Macroscopically the callus was loose and friable and was not pigmented. Histologically the tissue was composed almost entirely of large cells with central vacuoles and indistinct, peripherally situated nuclei (Figure 21). A small number of xylem-like cells were scattered throughout the callus, but no mature vascular elements were present. The intercellular spaces were large and empty. The cells at the periphery of each callus contained a few starch granules, which stained red with PAS. The centrally situated cells contained almost no starch.

After two days of growth on the medium supplemented with i^6Ado the tissue appeared macroscopically to be more compact. The histological appearances were consistent with this. There was a striking reduction in cell size together with the appearance of an amorphous material in the intercellular spaces. The tissue was more compact than tissue grown in the absence of cytokinin, and the intercellular spaces smaller.

During the first 12 days of growth on medium containing 9 μM i^6Ado there was a steady increase in the number of starch granules in the cells. After this time no further increase was observed in the number of starch granules per cell or in the number of cells in each section of tissue that contained starch granules.

After 14 days, nests of cells developed in the tissue. These nodules had the characteristics of Torrey's meristemoids (Torrey, 1966). That is, the cells were thinner walled, isodiametric, had prominent nuclei and no visible vacuoles. These regions were not observed to differentiate further during the course of the experiment (Figure 26).

Xylem-like cells were found scattered throughout the tissue during the course of the experiment (Figures 24,25). There was no

FIGURE 21

PHOTOMICROGRAPH OF KX-4/R1 CALLUS THAT HAD BEEN GROWN ON A MEDIUM WITHOUT CYTOKININ FOR 10 DAYS

This section was from the central area of a callus. It contains large undifferentiated cells. The nuclei are not visible in this section. Very few cells contain starch granules. When cells contain starch granules, the granules are situated peripherally, and appear as black dots. No vacuoles are visible in this preparation.

Stained with PAS. Magnification x 560.

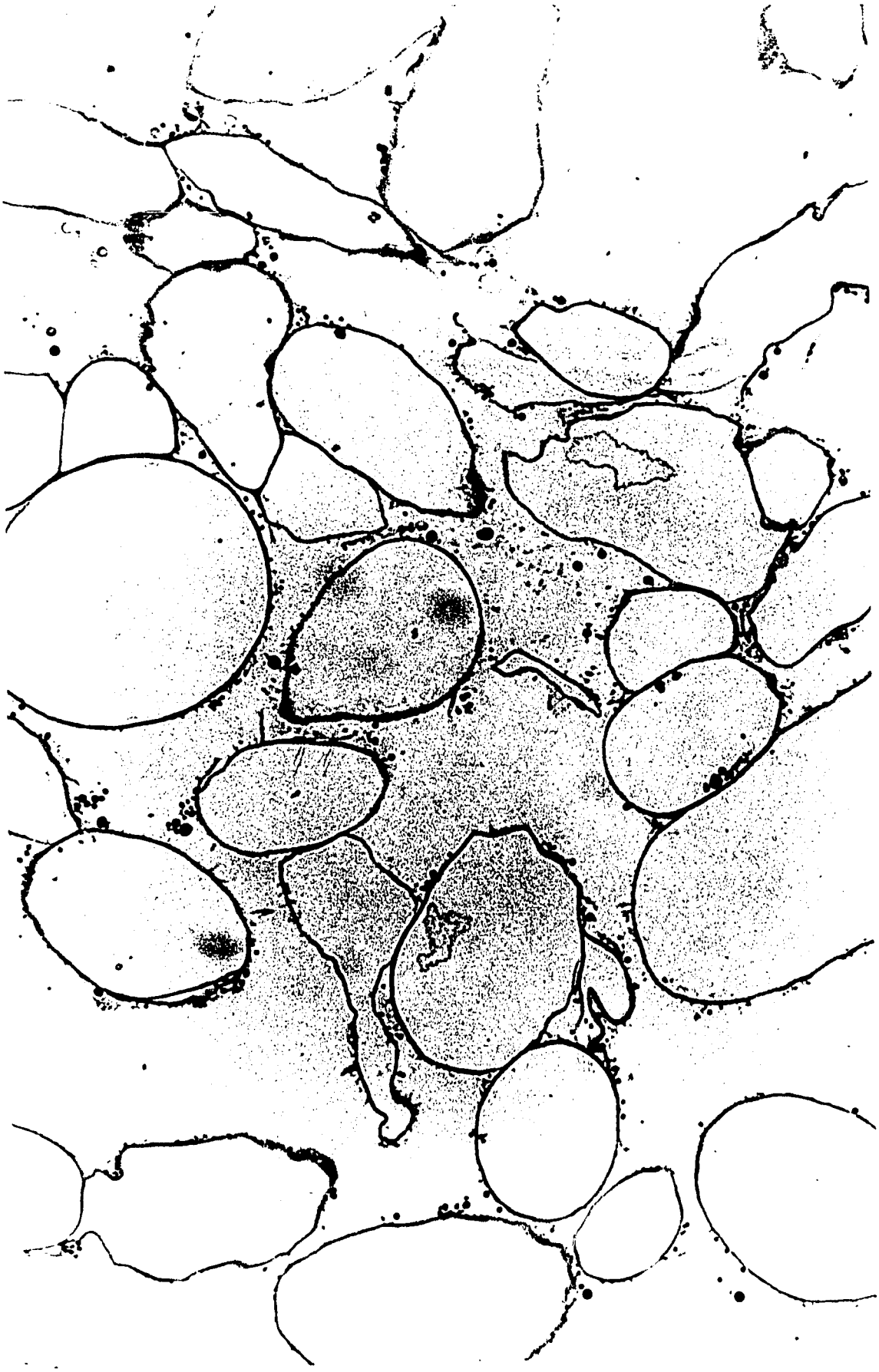


FIGURE 22

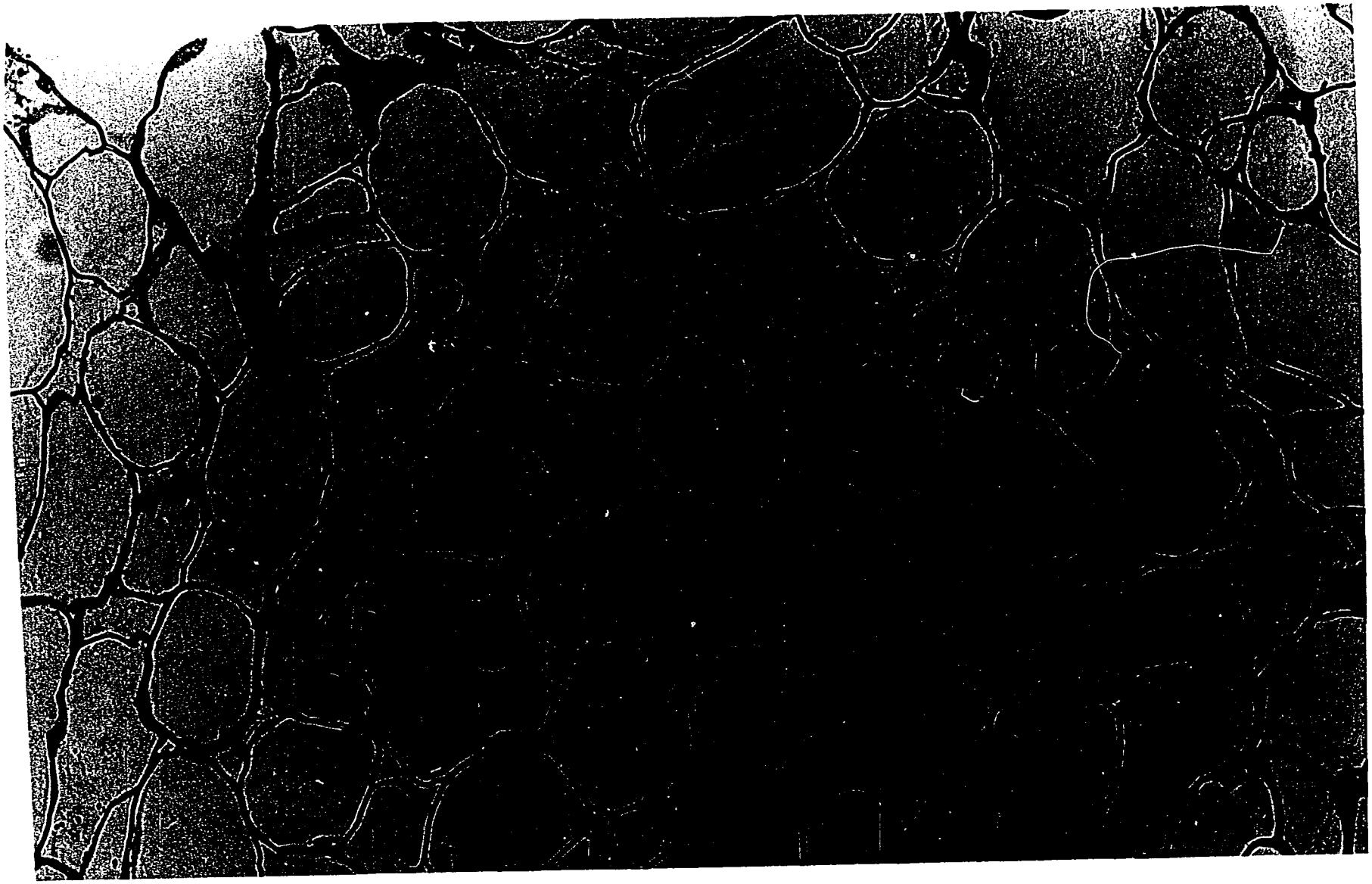
PHOTOMICROGRAPH OF KX-4/R1 TISSUE

GROWN FOR 4 DAYS IN THE PRESENCE OF 9 μM i^6Ado

- CENTER OF CALLUS

The cells from the center of the callus contain fewer starch granules and are larger than the cells at the periphery of the callus (Figure 23). Nuclei are not clearly visible in this section. The cells are considerably smaller than those that had been grown for 10 days on medium that did not contain cytokinin.

Stained with PAS. Magnification x 560.



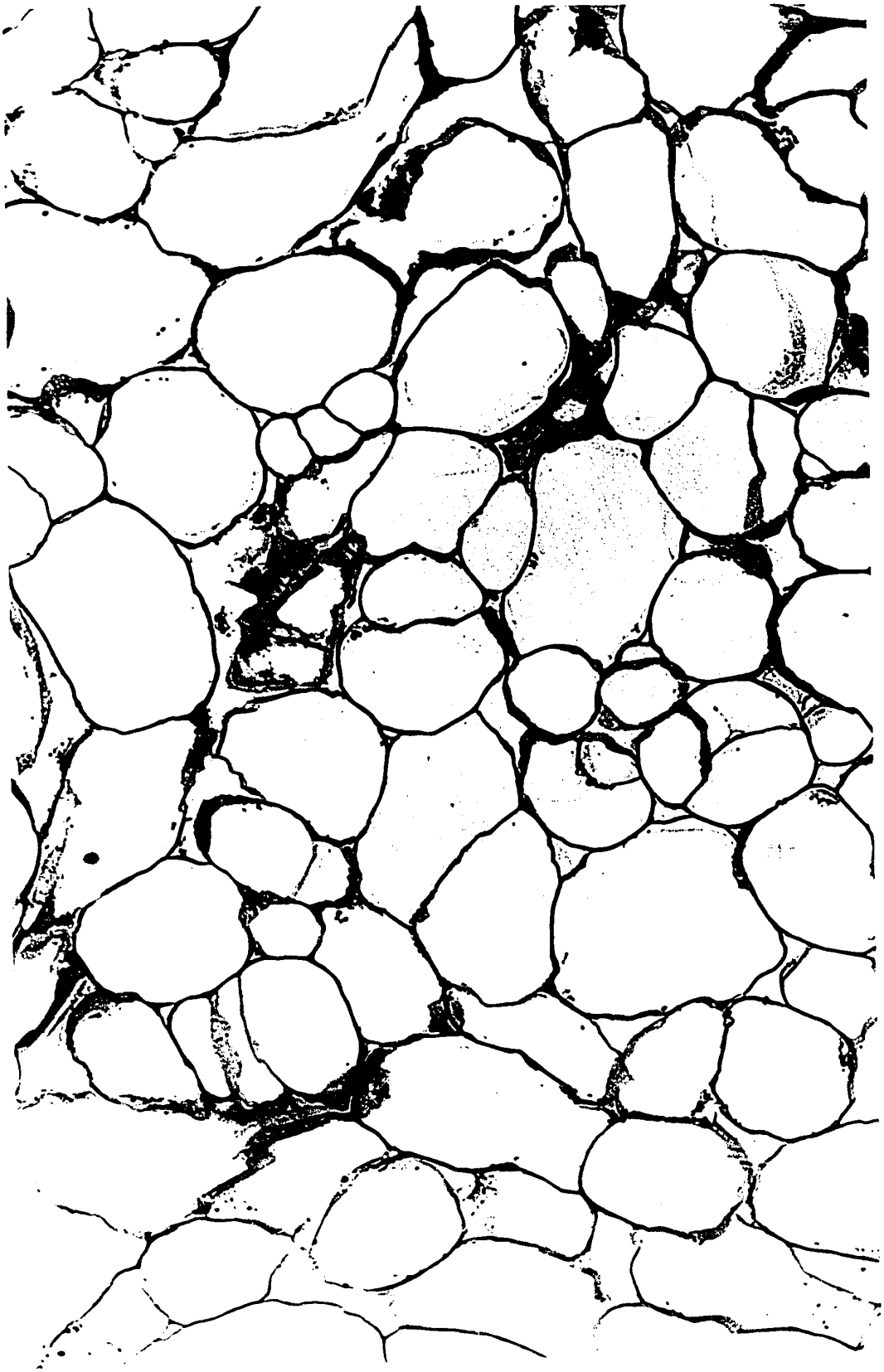


FIGURE 23

PHOTOMICROGRAPH OF KX-4/R1 TISSUE

TREATED WITH 9 μM i^6Ado FOR 4 DAYS

- PERIPHERY OF CALLUS

Cells are considerably smaller than in tissue grown in the absence of i^6Ado (Figure 21). Cells at the periphery of the callus are smaller than cells at the center of the callus (Figure 22). Many intracellular starch grains are visible and many of these are located in the region of the nuclei. One cell showing xylem-like secondary thickening is visible.(►)

Stained with PAS. Magnification x 560.

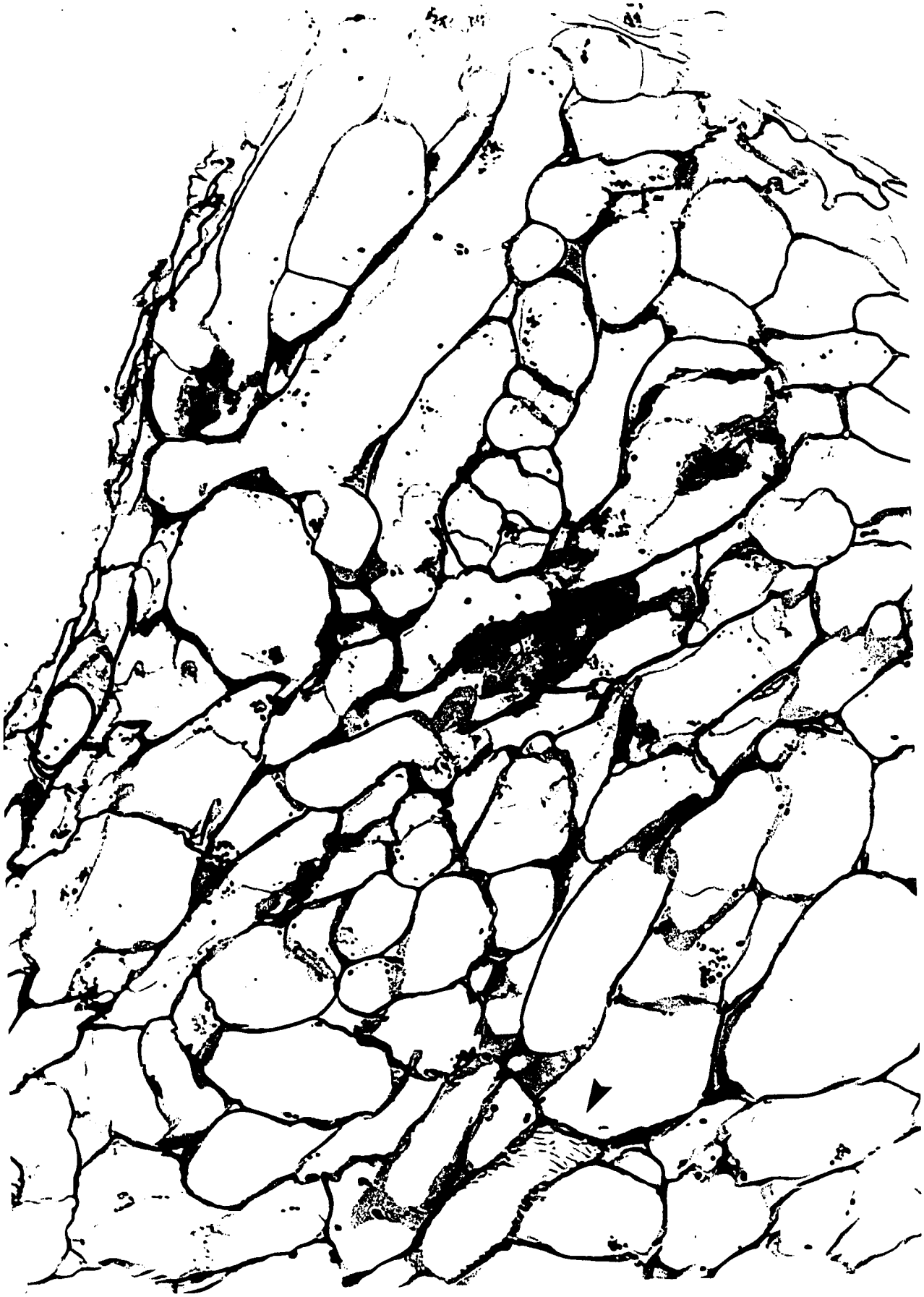
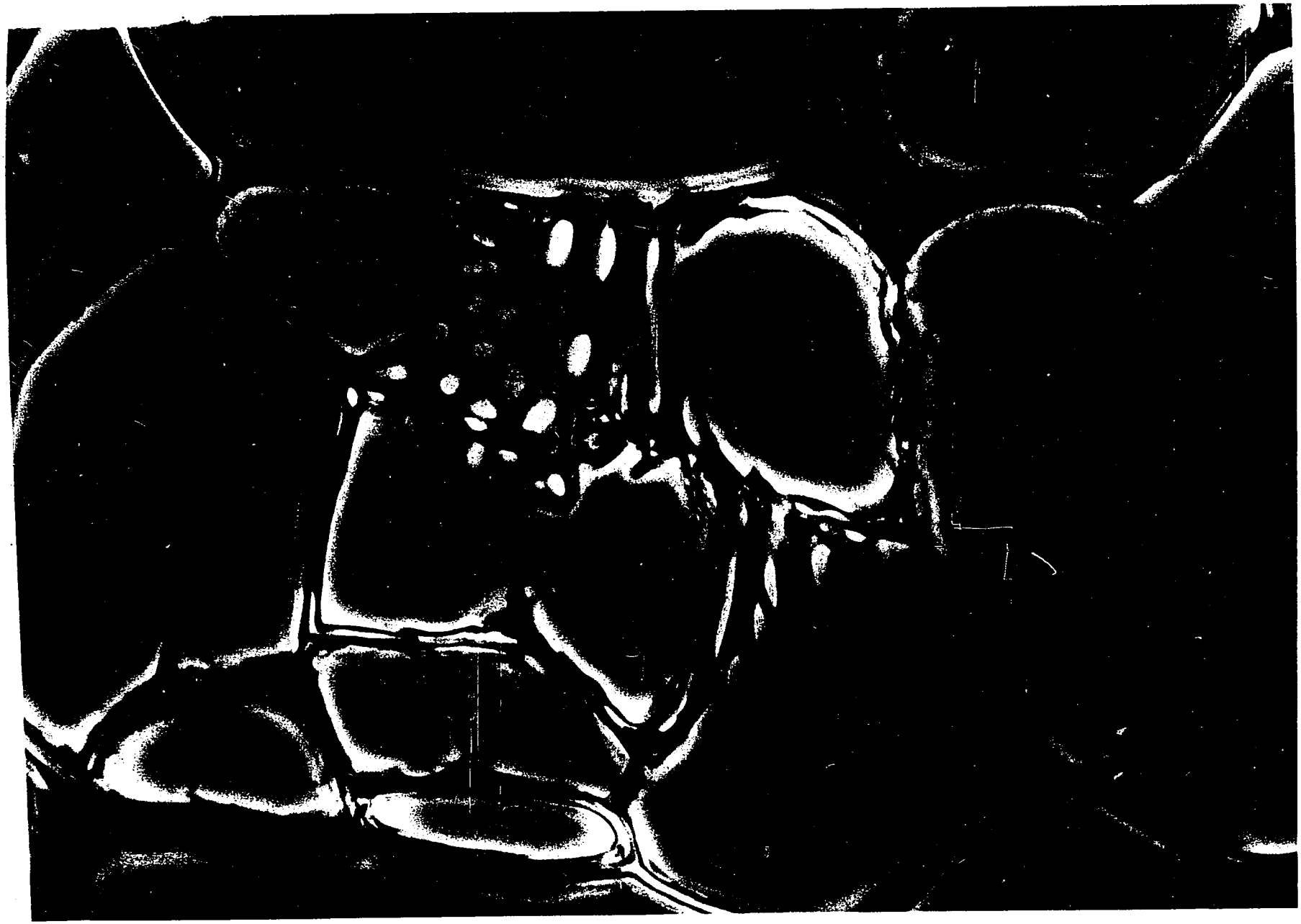


FIGURE 24

XYLEM-LIKE CELLS IN KX-4/R1 TISSUE
GROWN IN THE PRESENCE OF 9 μM i^6Ado

Xylem-like elements were sparsely scattered throughout tissue treated with 9 μM i^6Ado. This section was taken after 4 days growth in the presence of i^6Ado. Similar cells were visible in the tissue prior to treatment with i^6Ado.

Stained with toluidine blue. Magnification x 1400.



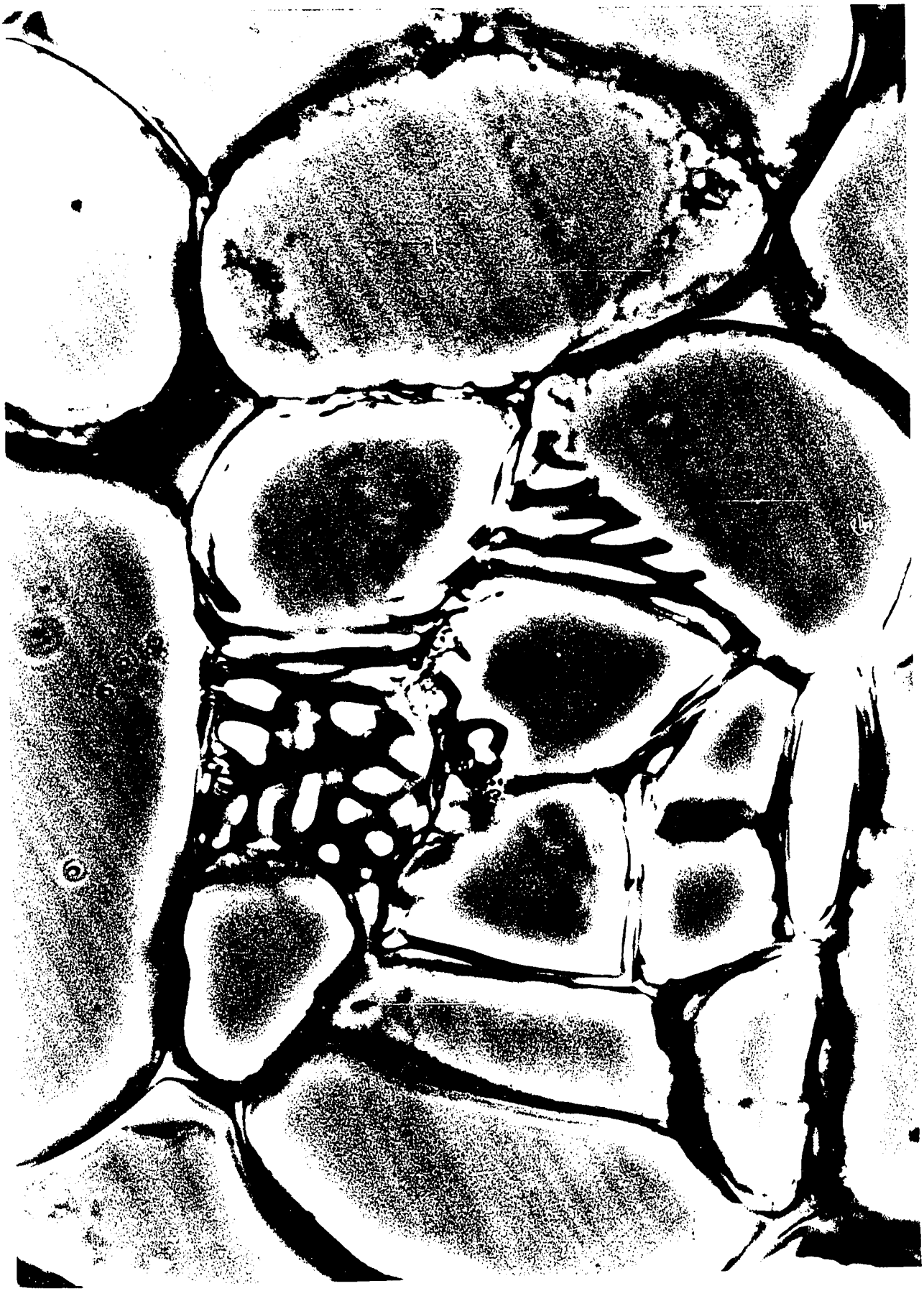


FIGURE 25

PHOTOMICROGRAPH OF KX-4/R1 TISSUE

TREATED WITH 9 μM i^6Ado FOR 16 DAYS

The cells in this section show secondary thickening similar to that observed in tissue treated with i^6Ado for 4 days. No mature vascular elements were seen after 16 days growth in the presence of i^6Ado, or at any other time interval. The number of xylem-like elements in the callus was constant throughout the course of the experiment (25 days).

Stained with toluidine blue. Magnification x 1400.

FIGURE 26

PHOTOMICROGRAPH OF KX-4/R1 TISSUE

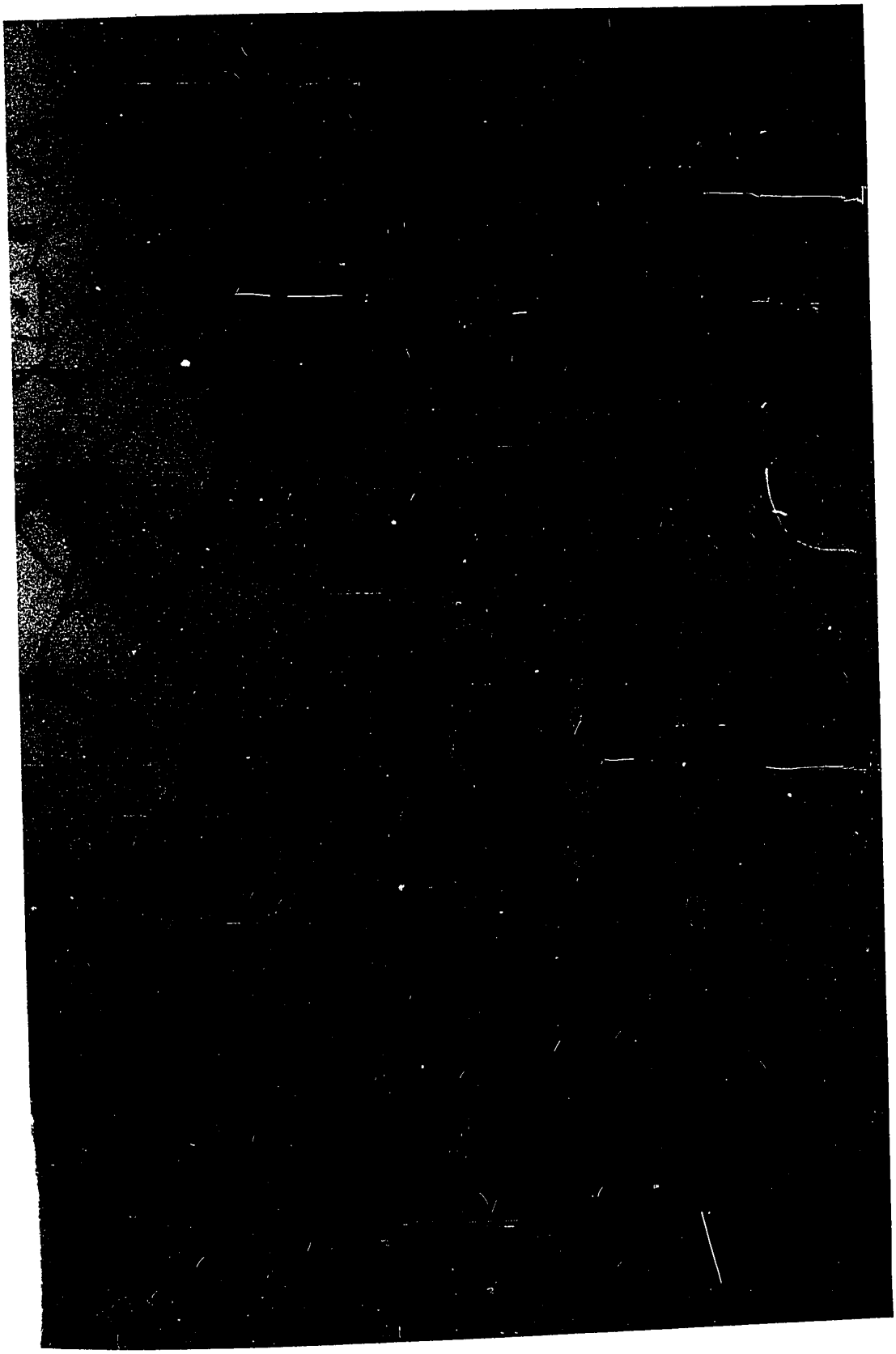
TREATED WITH 9 μM i^6Ado FOR 20 DAYS

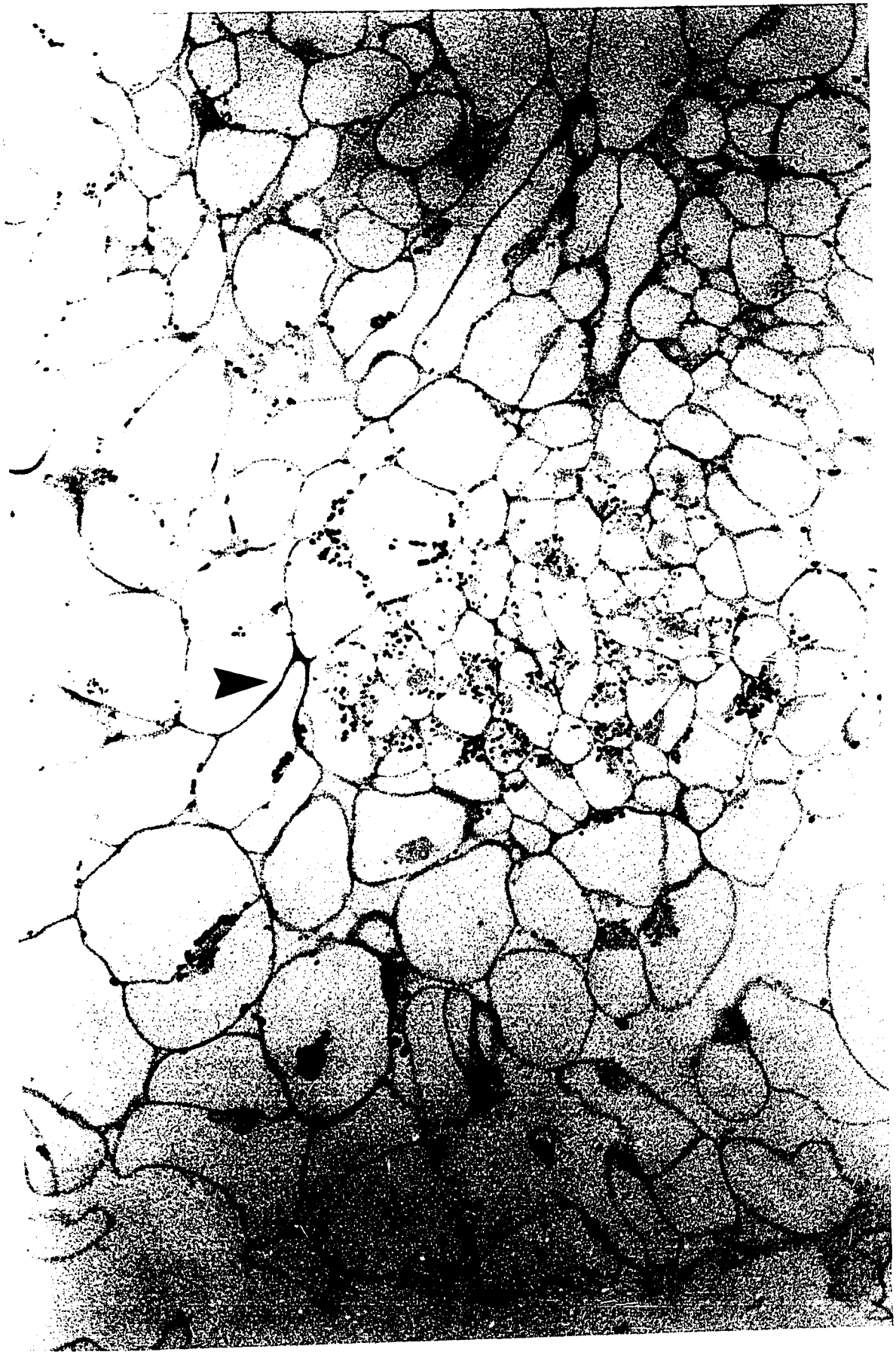
Starch grains are present in cells throughout this section. Many of the starch grains are located in the region of the nuclei. The nuclei are more prominent than in tissue at time 0, before the addition of i^6Ado to the growth medium (Figure 21), or in tissue that had been treated with 9 μM i^6Ado for a shorter time, e.g. 4 days (Figures 22 and 23). Many of the cells contain large vacuoles.

A meristemoid (page 116) is visible in this section. (►) It has characteristic small cells with prominent nuclei that are associated with large numbers of starch granules. The cells at the periphery of this section are more typical of the size of cells throughout the areas of callus that do not contain meristemoids.

A considerable quantity of amorphous material is present in the intercellular spaces.

Stained with PAS. Magnification x 560.





significant increase in the number of these cells, and at no time were mature vascular elements observed.

After 20 days, areas of white pigmentation appeared on the surface of the tissue. This pigmentation appeared similar to that developed by normal KX-4 tissue when treated with high concentrations of i^6Ado.

(b) Starch granules

During the first 12 days growth on medium containing i^6Ado the number of starch granules per cell increased by 400%. After 12 days the number of starch granules per cell remained constant. At no time did the number of granules per cell or the total number of granules per field decrease.

Initially, the starch granules were located at the periphery of each cell (Figure 21). During the course of the experiment the starch grains of increasing numbers of cells were situated round the nuclei (Figures 22,23,26).

Sections of tissue that had been grown in the absence of cytokinin for 10 days showed that almost all the starch granules were located in cells at the periphery of the callus. Cells in the centre of the callus contained few, if any, starch granules. During the course of the experiment the numbers of granules in the peripheral cells increased, and starch granules appeared in the centrally located cells (Figures 22,26). After 12 days, cells containing starch granules were distributed throughout the callus.

(c) Cell size and starch content

The most striking change in the tissue following transplantation to a cytokinin-containing medium was diminution in cell size. Two days

FIGURE 27

THE EFFECT OF i^6Ado ON CELL SIZE

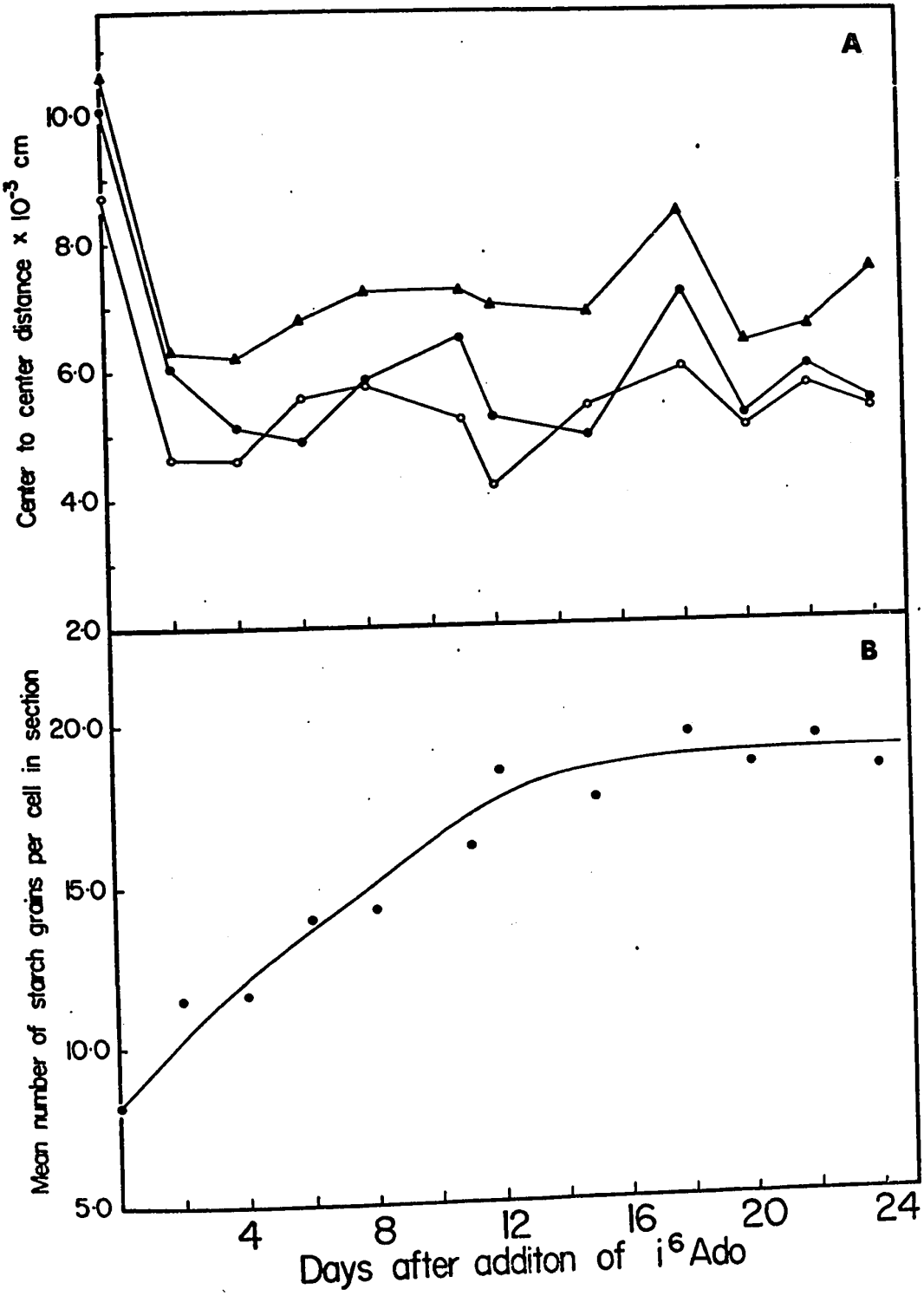
AND STARCH CONTENT OF KX-4/R1 TISSUE

KX-4/R1 tissue was grown in the absence of exogenous cytokinin for 10 days. Pieces of tissue each weighing 20 mg were then transferred to medium containing 9 μM i^6Ado. At this time, and at 2 to 3 day intervals subsequently, pieces of tissue were removed and examined histologically (page 48).

A. The mean center to center distance between two adjacent cells as a function of time.

- ▲ Both cells containing fewer than 10 starch grains per section.
- o Both cells containing more than 75 starch grains per section.
- ● One cell containing fewer than 10 and the other more than 75 starch grains per section.

B. The number of starch grains (stained with PAS) per cell as a function of time.


A
B
Center to center distance x 10^{-3} cm
10·0
8·0
6·0
4·0
2·0
Mean number of starch grains per cell in section
20·0
15·0
10·0
5·0
4
8
12
16
20
24
Days after additon of i^6Ado

after transfer to the cytokinin-containing medium the average cell diameter had decreased by approximately 40%. After this, the average cell size remained constant (Figure 27).

Cells which contained a large number of starch granules were smaller than those which contained few (Figure 27A). During the first 2 days of growth on a cytokinin-containing medium the diameter of both the cells rich in starch and cells with few starch granules, was reduced by approximately the same amount.

Growth of the callus

The growth of the callus, judged by an increase in wet weight of tissue, was stimulated by i^6Ado (Figure 28). In the absence of cytokinin the wet weight of the tissue increased only fourfold over 28 days. The weight increase of the callus was stimulated by the presence of i^6Ado in the growth medium. The fresh weight yield of the callus increased with increasing concentrations of i^6Ado up to 3 μM. Above this optimum concentration, the yield of tissue was slightly less than at 3 μM. However, even when the concentration of i^6Ado was increased to 9 μM the growth was 95% of the growth at the optimum concentration of i^6Ado.

These results contrast with the response of normal KX-4 callus tissue to i^6Ado treatment (Figure 20). The growth of the normal tissue is maximally stimulated by 0.6 μM i^6Ado. The concentrations of i^6Ado that produced the maximum growth of the KX-4/R1 variant tissue (3 μM) strongly inhibits the growth of normal KX-4 tissue.

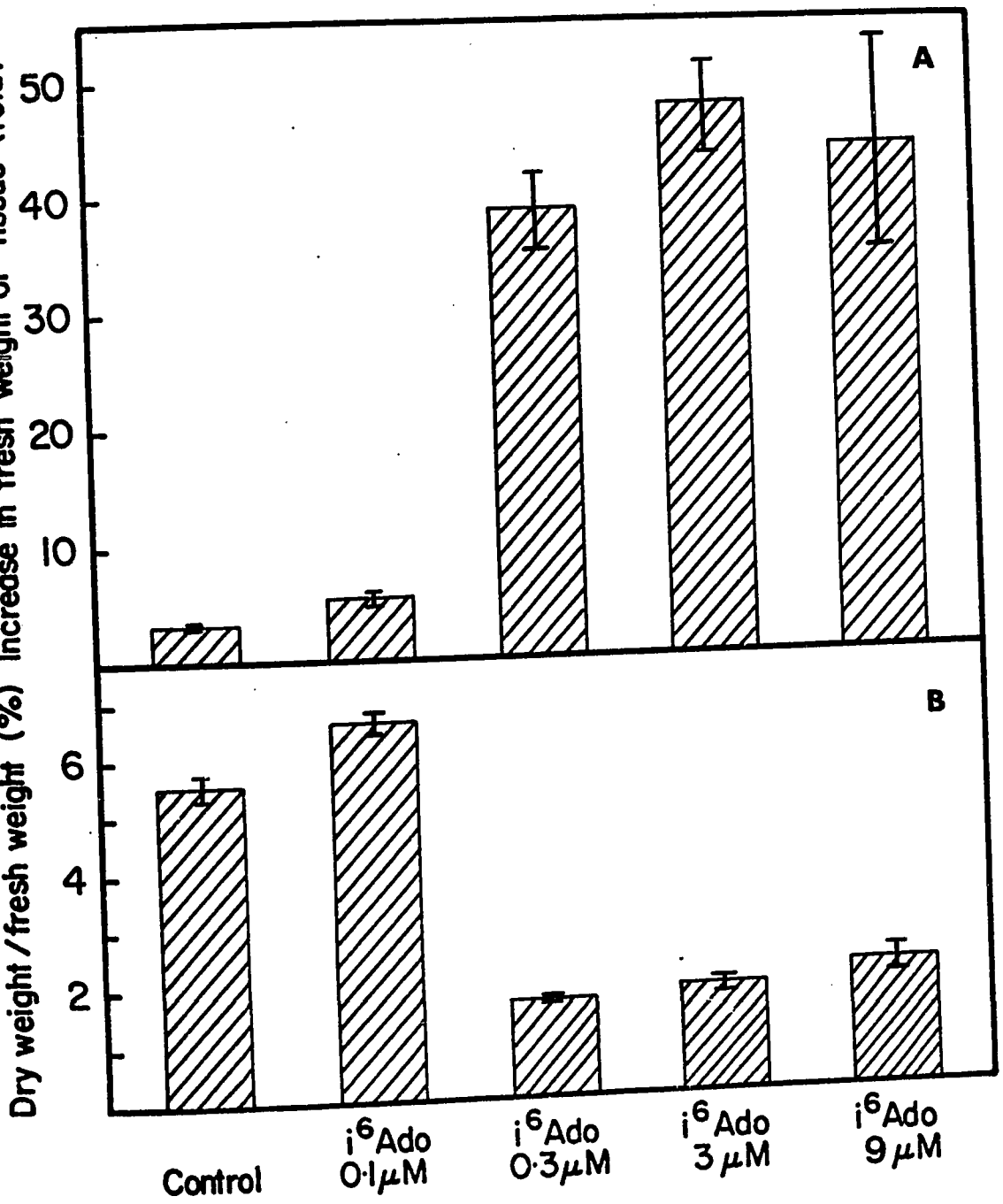
FIGURE 28

THE EFFECT OF i^6Ado ON THE GROWTH OF KX-4/R1 TISSUE

KX-4/R1 tissue was grown in the absence of exogenous cytokinin for 10 days. The callus was divided freehand in pieces each weighing approximately 15 mg. One hundred and twenty five pieces of callus were weighed at the beginning of the experiment to determine the initial fresh weight of the callus. Five pieces of callus were added to each of 25 conical flasks (250 ml volume). Each flask contained 100 ml of X medium. To four groups (each of 5 flasks) i^6Ado was added at concentrations of 0.1 μM, 0.3 μM, 3 μM and 9 μM. Each piece of callus was weighed at the end of the experiment, dryed (p. 111) and reweighed to determine the dry weight.

A. The mean increase in fresh weight of the tissue, ± the standard error of the mean expressed as a percentage of the initial weight of tissue.

B. The mean dry weight to fresh weight ratio of the tissue ± the standard error of the mean expressed as a percentage of the fresh weight at the end of the experiment.


A
B
50
40
30
20
10
6
4
2
Dry weight / fresh weight (%) Increase in fresh weight of tissue (fold)
Control
i⁶Ado 0·1μM
i⁶Ado 0·3μM
i⁶Ado 3μM
i⁶Ado 9μM

The origin of N^6-(Δ^2-isopentenyl)adenine in cultures of Corynebacterium fascians

The growth rate of C. fascians in liquid culture

Initial experiments were done to determine the growth characteristics of C. fascians under the conditions used in our laboratory. The bacteria were cultured as described on page 51, and the growth rate determined by ascertaining absorption of samples of the culture at 540 nm at intervals of 4 to 10 hours. During logarithmic growth phase the number of bacteria in the culture doubled in approximately 5 hours.

The uptake by C. fascians of [2 -^{14}C] mevalonic acid, [8 -^{14}C] adenine and [8 -^{14}C] adenosine

It was originally hoped that mevalonic acid would be taken up by the bacteria, and incorporated into the tRNA as the isopentenyl sidechain of i^6Ado as described by Fittler et al. (1968a), Peterkofsky (1968) and Kline et al. (1969). This would enable experiments to be done to determine the origin of the isopentenyl sidechain of i^6Ado that had been found in the C. fascians medium (Klambt et al., 1966; Helgeson and Leonard, 1966). Therefore, experiments were done to determine whether C. fascians incorporated [2 -^{14}C] mevalonic acid, [8 -^{14}C] adenine and [8 -^{14}C] adenosine when they were added to the culture medium. A culture of C. fascians in logarithmic growth phase had an absorbance of 1.35 A_{540} units. Aliquots of 5 ml of this culture were removed and spotted onto a Millipore filter of 0.45 μ pore size that was attached via an erlenmeyer flask to a vacuum pump. A small yellow spot of bacteria was trapped on the filter. The trapped bacteria were washed with 10 ml of culture medium over a period of 20 seconds. The filter

was then incubated for 2 minutes with medium containing the radioactive compound. The filter was washed with 10 ml of culture medium for 20 seconds and placed in a liquid scintillation vial and dried in an oven for 30 minutes at 50°C. Ten ml of toluene liquid scintillation cocktail I (page 41) were added to the vial, and the radioactivity on the filter determined by liquid scintillation counting.

In other experiments, both the initial wash with culture medium and the incubation with the medium containing the radioactive material was carried out at 0°C. This procedure showed the active uptake systems in the bacteria.

In some experiments an attempt was made to exchange radioactive material with 0.25 mM solutions of the corresponding unlabelled material that had been added to the culture medium. The bacteria-coated filter that had been incubated with the radioactive material for 2 minutes was washed with medium containing the unlabelled material for 3 minutes and subsequently washed with a further 10 ml of medium that did not contain the compound.

Both adenine and adenosine are taken up by the bacteria. The results of a typical experiment are shown in Table 5. After 2 minutes approximately 4.5% of the [8 -^{14}C] adenine that was present in the medium (6.6 μM) was associated with the bacteria. When the filter was incubated for 3 minutes with medium containing unlabelled adenine (0.25 mM) the amount of radioactivity associated with the bacteria on the filter was either unchanged or only very slightly reduced. When the experiment was performed at 0°C there was a reduction to 20% of the control value of the incorporation of radioactive [8 -^{14}C] adenine into the bacteria.

This indicated that [8 -^{14}C] adenine was incorporated into these cells and that this process was metabolically dependent. Using [8 -^{14}C] adenosine (9.5 μM) similar results were obtained (Table 5).

The specific activity of the [2 -^{14}C] mevalonic acid was much lower (2.95 μCi/μ mole) than that of the adenine (50 μCi/μ mole) or the adenosine (35 μCi/μ mole), and very little radioactivity was associated with the bacteria after incubation with [2 -^{14}C] mevalonic acid. Therefore, higher concentrations of [2 -^{14}C] mevalonic acid were used (0.14mM). Approximately 0.9% of the total radioactivity was associated with the bacteria following incubation for 2 minutes with 50 μM [2 -^{14}C] mevalonic acid (Table 5). This was much less than the radioactivity incorporated when adenine or adenosine were used. When the bacteria were incubated with [2 -^{14}C] mevalonic acid for 2 minutes, and then with 0.25 mM unlabelled mevalonic acid for 3 minutes, the radioactivity was one third of that found when the bacteria were not incubated with the unlabelled material. If the experiment were carried out at 0°C only 30% as much radioactivity was incorporated as when the experiment was carried out at room temperature. Data from two duplicate experiments are shown in Table 5. This indicated that much of the radioactivity associated with C. fascians that had been incubated with [2 -^{14}C] mevalonic acid was readily exchangeable with the unlabelled mevalonic acid that was subsequently added. The exchangeable radioactivity probably represented material adsorbed onto the bacteria rather than incorporated into their metabolic pools. It was possible that the small amount of radioactivity associated with the bacteria following incubation with [2 -^{14}C] mevalonic acid that is not exchanged with unlabelled

TABLE 5

THE UPTAKE OF [8 -^{14}C] ADENINE, [8 -^{14}C] ADENOSINE, AND [2 -^{14}C] MEVALONIC ACID BY C. FASCIANS

Aliquots (5 ml) of a culture of C. fascians that had an absorbance of 1.35 A_{540} units, were trapped on Millipore filters, 25 mm diameter, 0.45 μ pore size. Each filter was (i) washed for 20 seconds with 10 ml of medium, (ii) incubated for 2 minutes with 3 ml of medium that contained the radioactive material, and (iii) washed for 20 seconds with 10 ml of medium. The filters were placed in glass vials and dried for 30 minutes at 50°C. Ten ml of toluene scintillation cocktail I (page 41) was added to each vial, and the radioactivity on the filter determined by liquid scintillation counting.

In the second procedure of each group, steps (i) and (ii) were done at 0°C.

In the third procedure of each group, between steps (ii) and (iii), the filters were incubated for 3 minutes in medium that contained an 0.25 mM solution of the corresponding unlabelled material.

The table shows the results of two duplicate experiments.

TABLE 5

THE UPTAKE OF [8 - ^{14}C] ADENINE, [8 - ^{14}C] ADENOSINE AND [2 - ^{14}C] MEVALONIC ACID BY C. FASCIANS

A. [8 - ^{14}C] Adenine (50 μCi/μM)

	Wash 1 20 Seconds	Incubation 2 Minutes	Wash 2 3 Minutes	Wash 3 20 Seconds	% radioactivity incorporated
1.	10 ml medium	3 ml medium containing 6.6 μM [8 - ^{14}C] adenine		10 ml medium	4.6% 4.5%
2.	10 ml medium at 0°C	3 ml medium containing 6.6 μM [8 - ^{14}C] adenine at 0°C		10 ml medium	1.0% 0.8%
3.	10 ml medium	3 ml medium containing 6.6 μM [8 - ^{14}C] adenine	10 ml medium containing 0.25 mM adenine	10 ml medium	3.7% 3.9%

TABLE 5 (continued)

B. [8 - ^{14}C] Adenosine (35 μCi/μM)

	Wash 1 20 Seconds	Incubation 2 Minutes	Wash 2 3 Minutes	Wash 3 20 Seconds	% radioactivity incorporated
1.	10 ml medium	3 ml medium containing 9.5 μM [8 - ^{14}C] adenosine		10 ml medium	3.0% 2.9%
2.	10 ml medium at 0°C	3 ml medium containing 9.5 μM [8 - ^{14}C] adenosine at 0°C		10 ml medium	0.7% 0.8%
3.	10 ml medium	3 ml medium containing 9.5 μM [8 - ^{14}C] adenosine	10 ml medium containing 0.25 mM adenosine	10 ml medium	3.0% 3.1%

TABLE 5 (continued)

C. [2 - ^{14}C] Mevalonic Acid (2.95 μCi/μM)

	Wash 1 20 Seconds	Incubation 2 Minutes	Wash 2 3 Minutes	Wash 3 20 Seconds	% radioactivity incorporated
1.	10 ml medium	3 ml medium containing 0.14mM [2 - ^{14}C] mevalonic acid		10 ml medium	0.90% 0.87%
2.	10 ml medium at 0°C	3 ml medium containing 0.14mM [2 - ^{14}C] mevalonic acid at 0°C		10 ml medium	0.27% 0.29%
3.	10 ml medium	3 ml medium containing 0.14mM [2 - ^{14}C] mevalonic acid	10 ml medium containing 0.25mM mevalonic acid	10 ml medium	0.26% 0.24%

mevalonic acid represents actual incorporation of this material into the bacteria. Therefore experiments were done to determine whether [2 -^{14}C] mevalonic acid were incorporated into the tRNA of C. fascians in a manner analogous to that in other systems (Peterkofsky, 1968; Fittler et al., 1968a).

The incorporation of mevalonic acid into the tRNA of C. fascians

Two hundred and fifty ml of liquid medium containing 10 μCi of [2 -^{14}C] mevalonic acid (specific activity 2.95 μCi/μM) was inoculated with 10 ml of a culture of C. fascians in logarithmic growth phase.

The culture was aerated by shaking at 100 r.p.m. in a New Brunswick waterbath at 27°C for 22 hours. The bacteria were harvested by centrifugation for 10 minutes at 10,000 g at 4°C.

The bacteria (1 g) harvested from the radioactive culture were mixed with 5.5 g of unlabelled C. fascians that had been harvested previously. tRNA was extracted from the mixture of cells by the method described on page 38.

The tRNA was redissolved in 5 ml of distilled water. One ml and 2 ml of the RNA solution was placed in vials containing 15 ml of dioxane scintillation cocktail, and the radioactivity determined by liquid scintillation counting. The ultraviolet absorption spectrum of another aliquot was measured on a Cary 14 recording spectrophotometer to determine the quantity of tRNA. No radioactivity was associated with the tRNA. Thus [2 -^{14}C] mevalonic acid was not incorporated into the tRNA of C. fascians in quantities that could be detected using this technique, and therefore could not be used to label, in vivo, the Δ^2-isopentenyl sidechain of i^{6}Ado in the tRNA of C. fascians.

Incorporation of [8 -^{14}C] adenine into i^6Ade in C. fascians cultures

i^6Ade in cultures of C. fascians might originate by degradation of tRNA molecules containing i^6Ado, directly from adenine or a derivative, or from a compound that was not a derivative of adenine. In the latter case [8 -^{14}C] adenine added to the cultures would not be incorporated into i^6Ade released by the bacteria, whereas in the former cases it would be incorporated. Therefore, experiments were done to determine whether [8 -^{14}C] adenine is incorporated into i^6Ade in cultures of C. fascians.

From a 100 ml inoculum a 4 litre culture of C. fascians was grown in a 6 litre erlenmeyer flask. The culture was kept at 27°C in a rotary shaking waterbath (New Brunswick Scientific). Immediately after inoculation, and at 12, 36 and 60 hours later 12.5 μCi of sterile [8 -^{14}C] adenine (New England Nuclear, specific activity 50.5 μCi/μ mole) was added to the culture.

After 120 hours, the medium and the bacteria were separated by centrifugation for 10 minutes at 10,000 g at 4°C. The medium was filtered through a layer of Celite-545, 2.5 cm thick and 20 cm diameter. N^6-(Δ^2-Isopentenyl)adenine (0.2 mg) was added to the filtrate. The filtrate was adjusted to pH 3 with 1 N HCl, and applied to a Dowex 50W-X8 column, (H^+ form), 1 cm diameter x 15 cm length (page 31). The column was washed with 1 litre of distilled water, and eluted with 3 litres of 0.3 M NH_4OH. The eluate was evaporated to dryness on a rotary evaporator, and dissolved in 25 ml of distilled water. The aqueous material was extracted six times by shaking with equal volumes of

methylene chloride in a separatory funnel. The methylene chloride fractions were pooled, and reduced to 0.5 ml on a rotary evaporator. The material was streaked onto a 1 mm preparative silica gel T.L.C. plate (page 29) with an i^6Ade standard (0.05 mg) in another lane of the chromatogram. The chromatogram was developed using solvent D. The appearance of the chromatogram when viewed under ultraviolet illumination is shown in Figure 29.

The area corresponding to the i^6Ade was scraped off the plate, placed on filter paper in a filter funnel and the i^6Ade eluted with 50% aqueous ethanol. The eluate was reduced in volume. One quarter of this material contained 1500 cpm of ^{14}C as measured by liquid scintillation counting. The remaining 75% of the eluate of the area of the thin layer chromatogram that corresponded to i^6Ade was chromatographed on a 0.25 mm silica gel T.L.C. plate (page 29). N^6-(Δ^2-Isopentenyl)adenine was chromatographed in another lane of the chromatogram as a standard. The chromatogram was developed with solvent E and cut longitudinally in half. One half was divided into 1 cm strips that were placed in scintillation vials that each contained 10 ml of toluene scintillation cocktail II. The radioactivity of each strip was determined by liquid scintillation counting. Most of the radioactivity had the same Rf as i^6Ade (Figure 29). Some radioactivity had an Rf greater than that of i^6Ade in this system. The region that corresponded to the i^6Ade standard on the other half of the chromatogram was eluted with 50% aqueous ethanol, and divided into two parts.

One part was chromatographed on paper using solvent C. The chromatogram was cut into 1 cm strips. Each strip was placed in a vial

FIGURE 29

ISOLATION OF PUTATIVE i^6Ade FROM A CULTURE OF C. FASCIANS LABELLED WITH [8 -^{14}C] ADENINE

A culture of C. fascians was labelled with 50 μCi of [8 -^{14}C] adenine and treated as described on page 143. The material that was extracted into methylene chloride (page 143) was chromatographed on a preparative silica gel thin layer chromatogram, developed in solvent D.

A. Appearance of the chromatogram.

B. Rechromatography of the material that chromatographed with i^6Ade in A, on an analytical silica gel TLC plate developed with solvent E. One half of the plate was divided into 1 cm strips and the radioactivity of each strip determined by liquid scintillation counting.

C. The material that chromatographed with i^6Ade in B. above was eluted from the intact half of the chromatogram with 50% aqueous ethanol. Half this material was rechromatographed on paper, developed with solvent C.

The chromatogram was cut into 1 cm strips and the radioactivity of each strip determined by liquid scintillation counting.

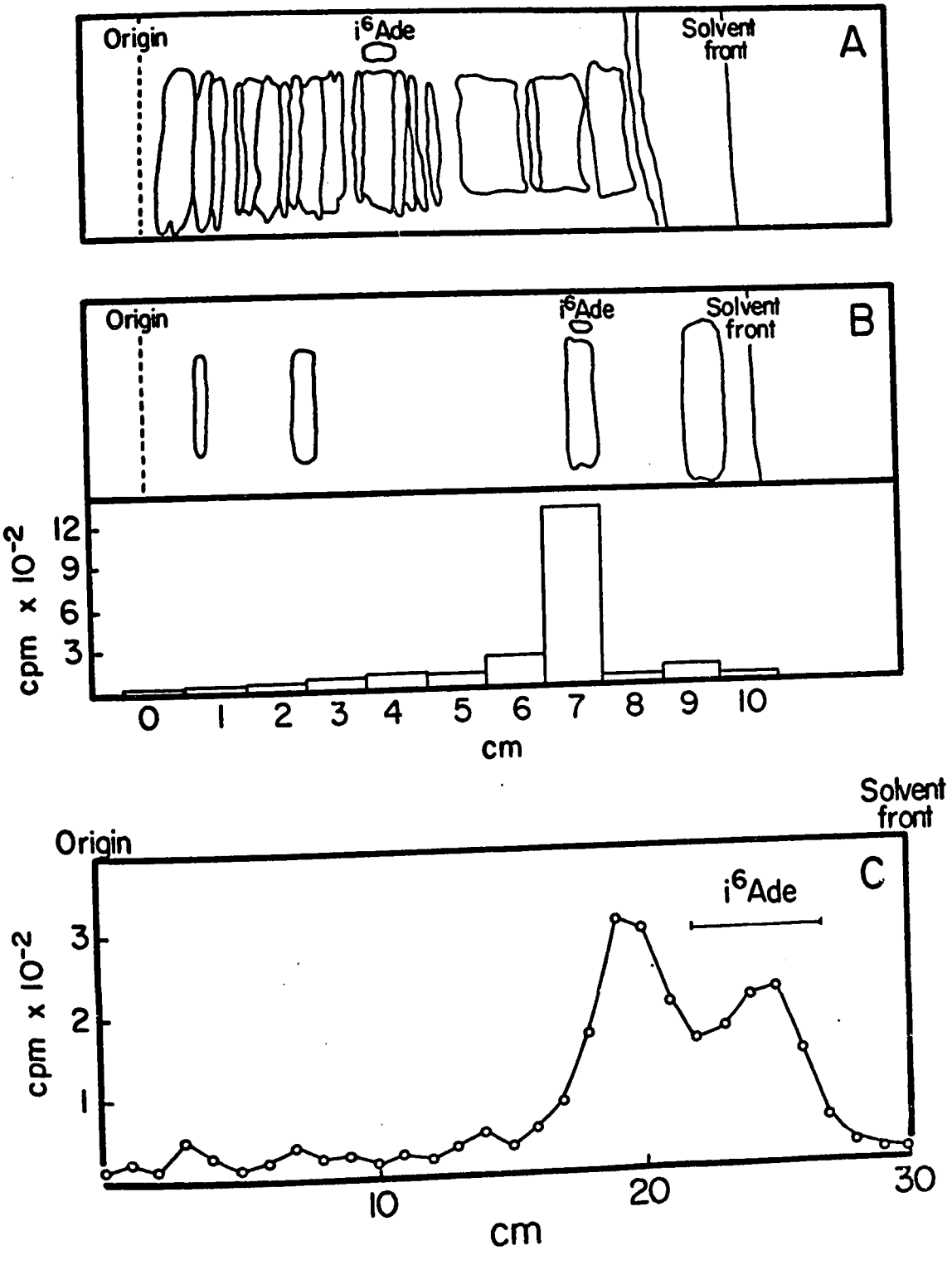

Origin
i⁶Ade
Solvent front
A
Origin
i⁶Ade
Solvent front
B
cpm x 10⁻²
12
9
6
3
0 1 2 3 4 5 6 7 8 9 10
cm
Solvent front
Origin
C
i⁶Ade
cpm x 10⁻²
3
2
1
10
20
30
cm

FIGURE 30

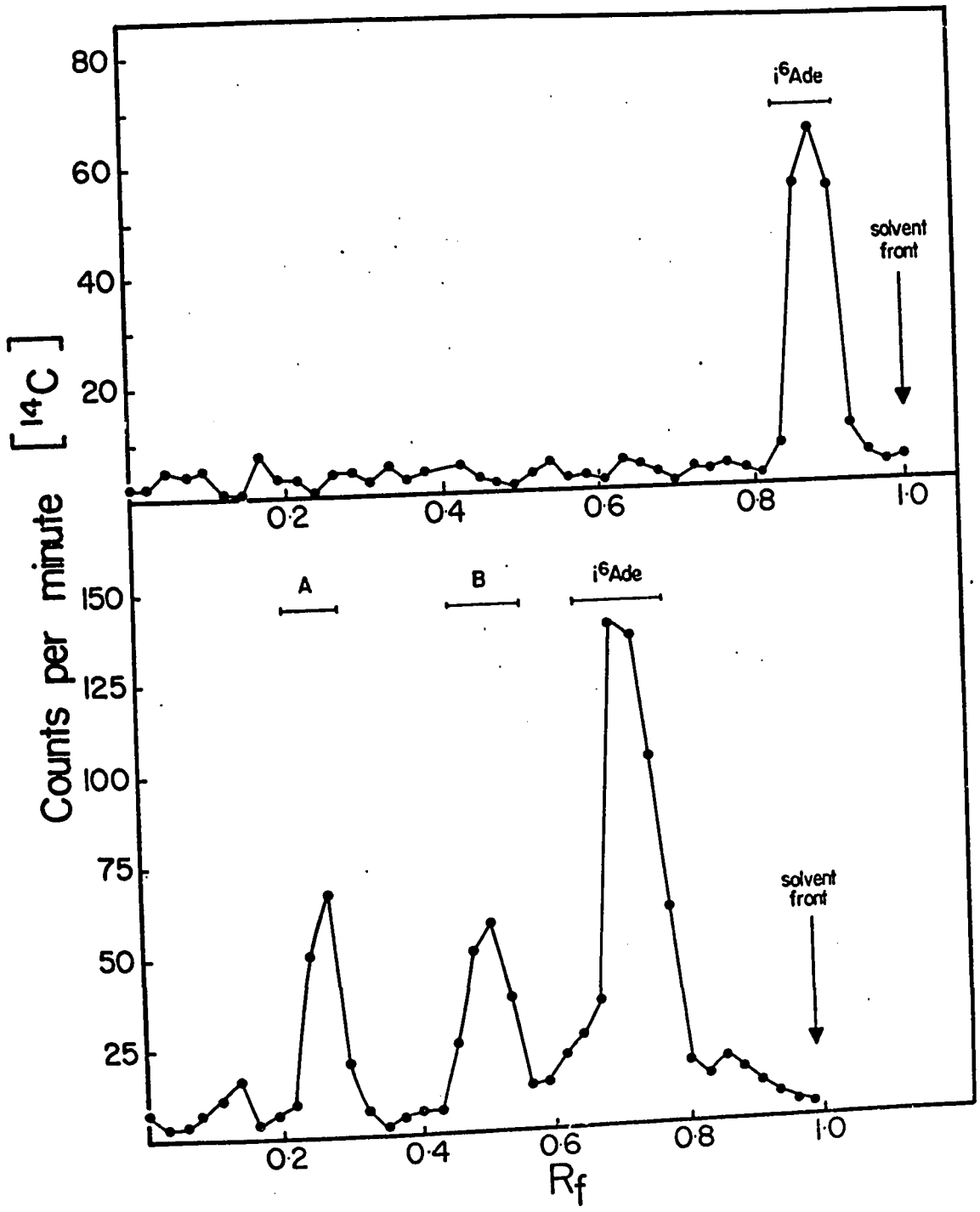
IDENTITY OF LABELLED MATERIAL FROM CULTURES OF C. FASCIANS THAT HAD BEEN LABELLED WITH [8 -^{14}C] ADENINE

Material that chromatographed with i^{6}Ade on thin layer chromatogram B (Figure 29) was rechromatographed on a partition column (Celite-545, page 32).

A. An aliquot of the fraction that contained i^{6}Ade was rechromatographed on paper using solvent C. The chromatogram was cut into 1 cm strips and the radioactivity of each strip was determined by liquid scintillation counting.

B. Another aliquot of the fraction that contained i^{6}Ade was hydrolysed with 1 N HCl for 15 minutes at 100°C. The material was rechromatographed on paper using solvent C. The chromatogram was cut into 1 cm strips and the radioactivity of each strip was determined by liquid scintillation counting.

A,B. Position of acid hydrolysis products of i^{6}Ade: 3H-7,7-dimethyl-7,8,9-trihydropyrimido-[2,1-i]purine and N^{6}-(3-hydroxy-3-methylbutyl) adenine.


80
60
40
20
i⁶Ade
solvent front
0·2
0·4
0·6
0·8
1·0
A
B
i⁶Ade
150
125
100
75
50
25
solvent front
Counts per minute [¹⁴C]
Rf

containing 10 ml of toluene liquid scintillation cocktail I. The radioactivity of each strip was determined by liquid scintillation counting. Radioactivity was distributed in two regions: some of the radioactivity chromatographed coincidentally with the i^6Ade standard, and some was found in a peak that had an Rf slightly less than i^6Ade in this system. The other part of the material that was eluted from the thin layer chromatogram was dried on a rotary evaporator. The material was redissolved in 5 ml of water saturated with ethyl acetate and chromatographed on a Celite-545 column (1.9 cm diameter x 50 cm length) (page 32). The first 35 ml fraction, in which i^6Ade and i^6Ado eluted, was collected and dried on a rotary evaporator. An aliquot of the material was hydrolysed with 1 N HCl (page 35). The products of acid hydrolysis were reduced to a small volume and chromatographed on paper developed in solvent C. The unhydrolysed part of the first 35 ml that eluted from the Celite column was chromatographed in another lane of the same chromatogram. The chromatograms were cut into 1 cm strips. Each strip was placed in a vial that contained 10 ml of toluene·cocktail II scintillation fluid. The radioactivity of each strip was determined by liquid scintillation counting. The distribution of radioactivity on each chromatogram is shown in Figure 30. In the unhydrolysed sample, all the radioactivity chromatographed coincidentally with i^6Ade. In the chromatogram of the acid hydrolysed sample all the radioactivity chromatographed with the products of acid hydrolysis of i^6Ade.

I concluded that [8 -^{14}C] adenine was incorporated into [8 -^{14}C] i^6Ade liberated into the medium by the bacteria.

The efficiency of recovery of i^6Ade from cultures of C. fascians

To make quantitative measurements of the production of [8 -^{14}C] i^6Ade by these bacteria it was important to ascertain the recovery of i^6Ade from the C. fascians medium. A small quantity of a solution of unlabelled i^6Ade, the exact concentration of which had been determined by measuring its absorbance at 269 nm, was added to the culture immediately before commencing the extraction procedure. The absorbance of the i^6Ade fraction at 269 nm was determined again when the extraction procedure was completed. It was assumed that the endogenously produced i^6Ade made an insignificant contribution to the absorbance at 269 nm compared with the quantity of i^6Ade added to the medium. Thus by comparing the absorbance of the i^6Ade before and after the extraction procedure, the recovery could be determined. In each case between 25% and 35% of the i^6Ade was recovered after the extraction procedure.

The optimum procedure used to label the i^6Ade produced by C. fascians with [8 -^{14}C] adenine

It had been suggested by Klambt et al. (1966) that C. fascians cultures liberate the majority of the i^6Ade late in logarithmic growth phase or in stationary phase. Therefore, it was possible that the time at which [8 -^{14}C] adenine was administered to the cultures would be important in determining the amount of radioactivity found in the i^6Ade liberated by the bacteria. The effect of time of administration of [8 -^{14}C] adenine on the quantity of label incorporated into i^6Ade was therefore determined.

Two cultures of C. fascians were grown under identical conditions and grew at the same rate. One was labelled with 5 μCi of [8 -^{14}C] adenine 18, 42, 48 and 65 hours after inoculation of the culture, whereas the other was given the same total dose of [8 -^{14}C] adenine 65 hours after inoculation. One hundred and twenty hours after inoculation both of the cultures had reached the stationary growth phase. The bacteria were separated from the medium by centrifugation for 10 minutes at 10,000 g at 4°C. Both cultures were treated identically during the extraction procedure. N^6-(Δ^2-Isopentenyl)adenine (0.2 mg) was added to each lot of medium to determine the recovery of [8 -^{14}C] i^6Ade. The medium was filtered through Celite. The filtrate was reduced to one sixth of its original volume in vacuo at 20°C on a rotary evaporator. The concentrated medium was extracted for 24 hours with methylene chloride in a liquid-liquid extractor. The methylene chloride was evaporated to dryness at 20°C. The residue was redissolved in 5 ml of water saturated with ethyl acetate and chromatographed on a Celite column developed with solvent A. The initial 35 ml fraction, in which i^6Ade elutes, was evaporated to dryness at 20°C. The residue was dissolved in 200 μl of 35% aqueous ethanol and applied to a paper chromatogram that was developed using solvent C. The distribution of radioactivity on the chromatogram was determined by means of an Actigraph III radiochromatogram scanner. The i^6Ade was eluted from the paper chromatogram and the ultraviolet absorption spectrum of the eluent was determined using a Cary - 14 spectrophotometer. The recovery of an i^6Ade standard from each culture was determined as described on page 150. The recovery of the i^6Ade in the experiment in which [8 -^{14}C] adenine was given at one time

FIGURE 31

A COMPARISON OF THE QUANTITY OF RADICACTIVITY ASSOCIATED WITH i^6Ade EXTRACTED FROM CULTURES OF C. FASCIANS LABELLED WITH [8 -^{14}C] ADENINE IN SINGLE AND DIVIDED DOSES

A. Putative i^6Ade was extracted from a culture of C. fascians that had been labelled with four lots of 5 μCi of [8 -^{14}C] adenine, as described on page 151, except that chromatography on LH-20 was replaced by paper chromatography, in solvent C.

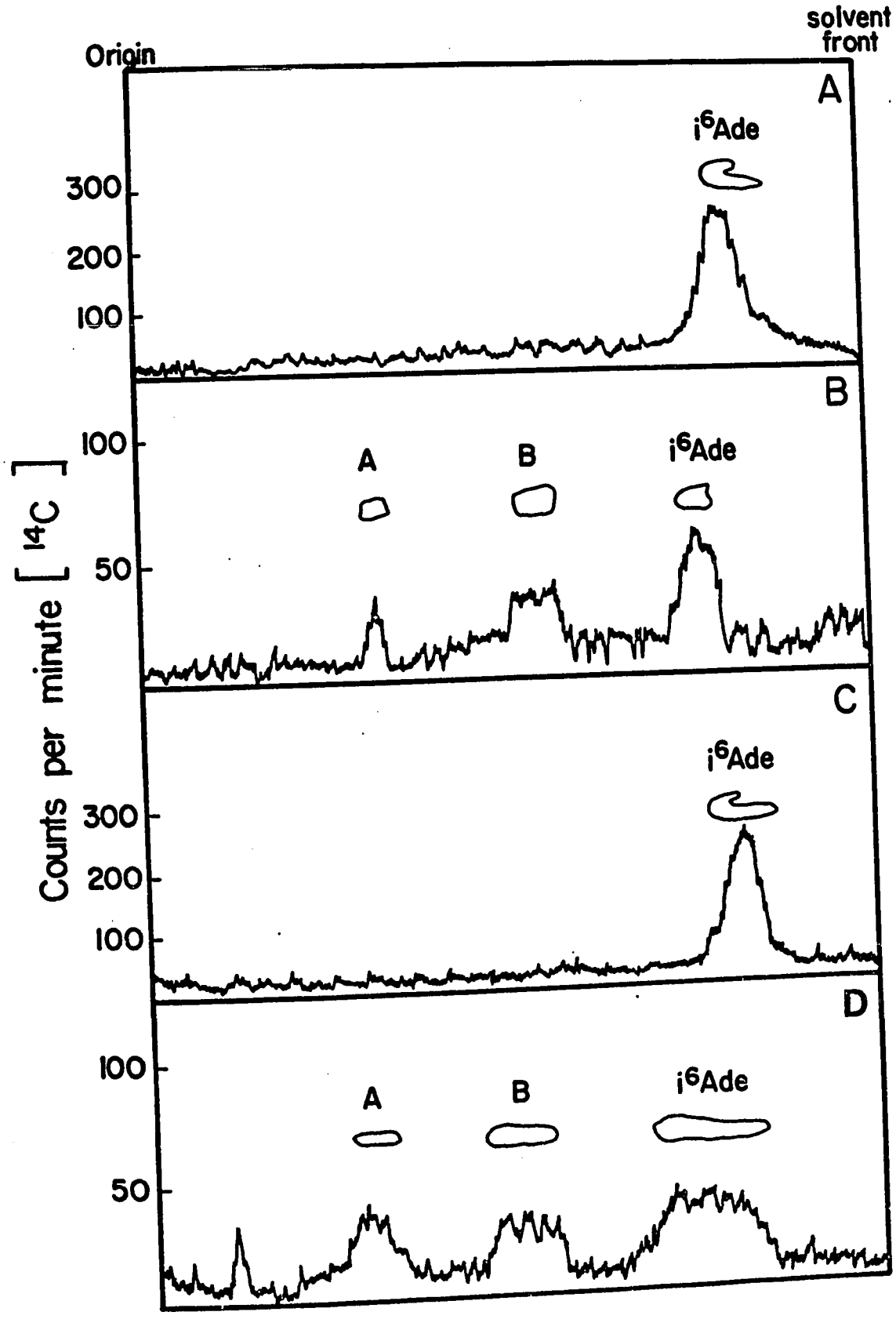
B. The material that chromatographed with i^6Ade on paper was eluted with water and hydrolysed with 1 N HCl for 15 minutes at 100°C. The product was chromatographed on paper developed with solvent C.

C. Putative i^6Ade was extracted from a culture of C. fascians that had been labelled with 20 μCi of [8 -^{14}C] adenine as described on page 151. Subsequent procedures were done as in A. above.

D. The material that chromatographed with i^6Ade on paper in C. above was hydrolysed with 1 N HCl for 15 minutes at 100°C. The product was chromatographed on paper developed with solvent C.

In each case the distribution of radioactivity on the chromatogram was determined using a radiochromatogram scanner.

A,B. Position of products of acid hydrolysis of i^6Ade: 3H-7,7-dimethyl-7,8,9-trihydropyrimido-[2,1-i]purine and N^6-(3-hydroxy-3-methylbutyl) adenine.


Origin
solvent front
A
i6Ade
300
200
100
B
100
50
A
B
i6Ade
C
i6Ade
300
200
100
D
100
50
A
B
i6Ade
Counts per minute [14C]

was 32% and in the other experiment it was 29%. The quantity of [8 -^{14}C] i^{6}Ade in the medium was the same in each case.

As further evidence that the radioactive material was i^{6}Ade, the material that chromatographed with i^{6}Ade on paper in solvent A was hydrolysed for 15 minutes with 1 N HCl at 100°C and chromatographed on paper in solvent C. The distribution of radioactivity on the chromatogram determined by scanning the chromatograms with a radiochromatogram scanner was identical with the regions of ultraviolet absorption of the i^{6}Ade marker and its characteristic products of acid hydrolysis (Figure 31).

I concluded that either method of administration: divided doses throughout the growth phase or a single dose late in logarithmic growth phase, resulted in similar incorporation of label into the i^{6}Ade liberated by the bacteria.

The effect of heating and acidification of C. fascians cultures on the amount of i^{6}Ade extracted from the cultures

The quantity of [8 -^{14}C] i^{6}Ade recovered from C. fascians cultures in the experiments described above was small, even when the cultures were labelled with 20 μCi of [8 -^{14}C] adenine. Klambt et al (1966) estimated that cultures of C. fascians contained 100 μg per litre of i^{6}Ade.

The results of the initial experiments indicated that [8 -^{14}C] adenine was rapidly incorporated into C. fascians, and that it was a precursor of i^{6}Ade. It appeared, therefore, that there was a discrepancy between the quantity of i^{6}Ade that Klambt et al. (1966) had estimated to be present in C. fascians cultures, and the quantity of radioactivity incorporated into i^{6}Ade in these experiments. A possible explanation for this was that a heating and acidification step had been

used by Klambt et al. (1966) at the beginning of the extraction procedure. Therefore, experiments were done to determine the effect of this step on the quantity of i^6Ade extracted from the cultures.

A culture of C. fascians (2 litres or 4 litres) was grown to stationary phase and divided into two equal parts.

One part was extracted by Procedure 1. This procedure included the heating and acidification steps used by Klambt et al. (1966). The other half of the culture was extracted by the method described on page 60 - Procedure 2.

Procedure 1

The culture was heated for 90 minutes at 95° in a waterbath, and then acidified with 16 ml of concentrated hydrochloric acid per litre. The culture was allowed to cool for 30 minutes so that the bacteria precipitated leaving a clear supernatant. The supernatant was decanted and the precipitate washed with 35% aqueous ethanol, filtered and discarded. The washings and supernatant were pooled, filtered through Celite, neutralized with 1 N NH_4OH and reduced to 250 ml on a rotary evaporator in vacuo. The extract was then treated in exactly the same manner as the cultures that were subjected to Procedure 2.

Procedure 2

The culture was extracted as described on page 60, except that the first 125 ml of eluate from the Celite column was evaporated to dryness. This fraction will contain, if present, i^6Ade, i^6Ado, zeatin and ribosyl zeatin. The residue was redissolved in 2 ml of 35% aqueous ethanol, and chromatographed on an LH-20 column (2.5 cm diameter x 50 cm length) developed with 35% aqueous ethanol. The elution profiles from

this column procedure are highly reproducible. The level of i^6Ade and/or derivatives extracted from the cultures was below that which could be recorded spectrophotometrically. However, Figure 34C shows the resolution of a synthetic mixture of four cytokinins (0.2 mg each) including i^6Ade on this column.

The eluates from the column separations of the two extracts obtained from Procedures 1 and 2 were divided into six fractions, four of which corresponded to the positions of the four standard cytokinin compounds i^6Ade, i^6Ado, zeatin and ribosyl zeatin, and one immediately before the first peak and one immediately after the last peak. Each fraction was evaporated to dryness *in vacuo*.

The TMS derivative of each dried fraction was prepared as described in the Methods section. The TMS derivative was then chromatographed on a GLC column as described in the Methods section. Zeatin, ribosyl zeatin and i^6Ado were not detected in the corresponding fractions from the LH-20 column, and no material that corresponded to any known cytokinin was detected in the first and last fractions of the series.

However, small quantities of i^6Ade were detected in the corresponding LH-20 column fraction. The results of a typical experiment are shown in Figure 32.

It is estimated that approximately 0.5 to 1 μg of i^6Ade per litre of *C. fascians* culture were extracted using Procedure 2, and 10 to 12 μg per litre using Procedure 1, that involved heating and mild acidification. The results obtained using Procedure 1 are quantitatively similar to those of Klambt *et al.* (1966) who also used a mild heating and acidification step. An estimate of the quantity of i^6Ade

FIGURE 32

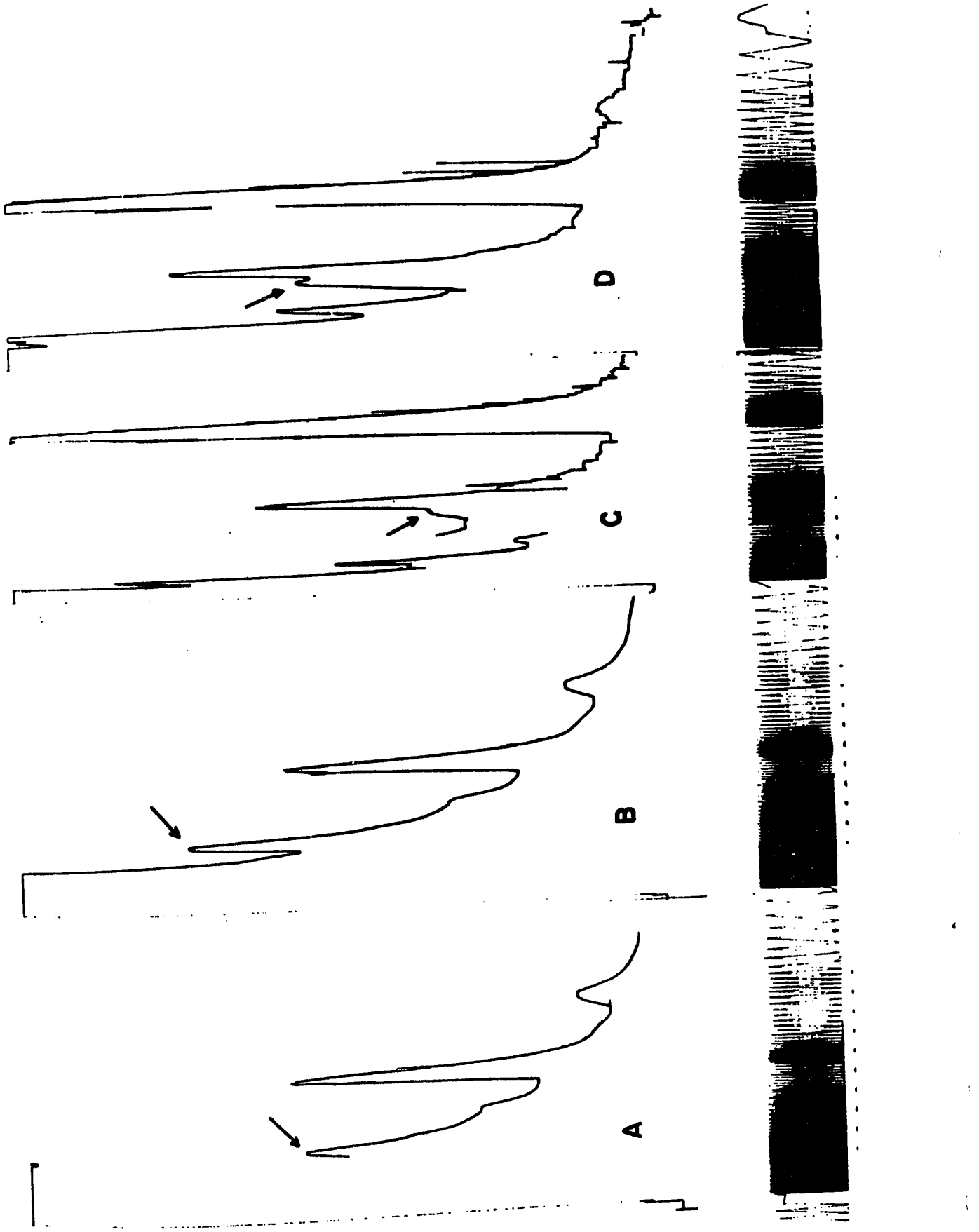
GAS CHROMATOGRAPHY OF EXTRACTS OF C. FASCIANS CULTURES

A. Sample obtained by Procedure 1. An aliquot of 1.5 µl of a total of 20 µl containing the TMS derivative of the entire sample obtained from the LH-20 column fraction corresponding to i^6Ade. Attenuation, 16.

B. Same as A. except with coinjection of 1 µl of a TMS derivative prepared from 20 µg of i^6Ade, final volume of solution 20 µl. Attenuation, 16.

C. Sample obtained by Procedure 2. An aliquot of 0.5 µl of a total of 20µl containing the TMS derivative of the entire sample obtained from the LH-20 column fraction corresponding to i^6Ade. Attenuation, 8.

D. Same as C. except 1.0 µl aliquot used and sample coinjection with 0.2 µl of the TMS derivative of authentic i^6Ade. (Prepared same as in B.) Attenuation, 16.


D
C
B
A

present in each culture at the beginning of the solvent extraction procedure can be estimated because the yield of the solvent extraction procedure was 25 to 35% (page 150).

Extraction of C. fascians cultures labelled with [8 -^{14}C] adenine

Further support for the quantitative differences in the amount of i^{6}Ade extracted from the cultures via Procedures 1 and 2 was obtained using a radioactive labelling technique. To a culture of C. fascians (1 litre) [8 -^{14}C] adenine was added in 10 μCi portions 6, 12, 20 and 26 hours after the culture was inoculated. Thirty hours after the last aliquot of [8 -^{14}C] adenine, four A_{269} units of i^{6}Ade was added and the culture was divided into two equal parts. One part was extracted according to Procedure 1 and the other according to Procedure 2. The amount of i^{6}Ade recovered from the LH-20 fractions via each of the two procedures was determined by the spectrophotometric analysis. The fractions were then evaporated to dryness and the residues were dissolved in 200 μl of 95% ethanol. The samples were chromatographed on paper in solvent B.

The radioactivity profiles of each chromatogram showed a single radioactive spot migrating coincidentally with i^{6}Ade. The amount of radioactivity (adjusted for the percentage recovery; 25%) was: Procedure 1, 15,200 cpm; Procedure 2, 2,740 cpm.

No radioactive zeatin, ribosyl zeatin, nor i^{6}Ado was detected in the respective LH-20 fractions from these columns.

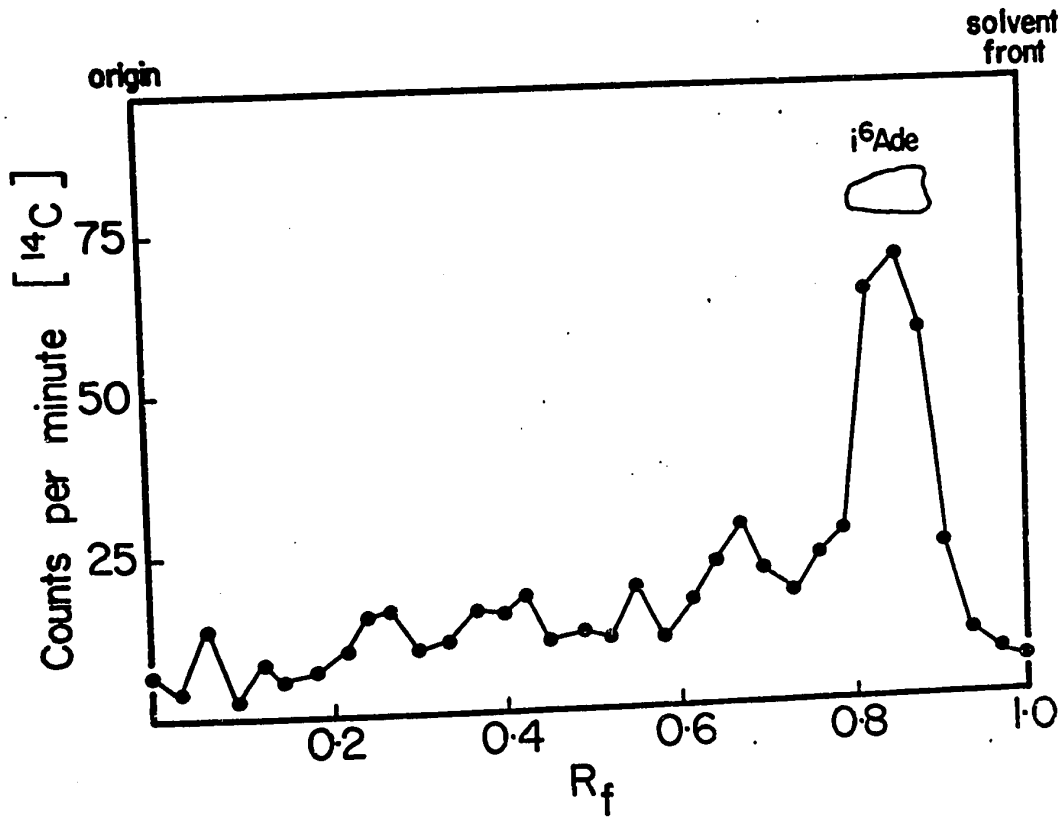
Acid hydrolysis of C. fascians tRNA

In view of the above results, it seemed that heating and subse-

FIGURE 33

PAPER CHROMATOGRAM OF N^6-(Δ^2-ISOPENTENYL)ADENINE FROM C. FASCIANS tRNA

tRNA was extracted from C. fascians that had been grown in the presence of [8 -^{14}C] adenine as described in the text. The tRNA was treated with 0.2 N HCl as described on page 162. The reaction mixture was chromatographed on a Celite column (1.9 cm diameter x 50 cm length) using system A. The first 15 ml of eluate was collected and the contents were chromatographed together with an authentic sample of i^6Ade (0.1 mg), on paper developed in solvent C. The chromatogram was cut into 1 cm strips and the radioactivity of the strips was determined by liquid scintillation counting.


origin
solvent front
i⁶Ade
Counts per minute [¹⁴C]
75
50
25
0·2
0·4
0·6
0·8
1·0
R_f

quent acidification of C. fascians cultures prior to extraction could release i^6Ade from the tRNA. This point was tested further. One hundred ml cultures of C. fascians were labelled with 5 μCi portions of [8 -^{14}C] adenine 6, 18, 30 and 42 hours after inoculation. The bacteria were harvested after 60 hours incubation by centrifugation for 10 minutes at 10,000 g at 4°C. The sample was added to 10 g of unlabelled C. fascians cells harvested previously. tRNA was extracted and purified. To simulate the extraction conditions (Procedure 1) used for cultures of C. fascians, tRNA was dissolved in 2 ml of 0.2 N hydrochloric acid and the solution placed in a waterbath, previously heated to 98°C. After the solution had warmed to 95°C it was allowed to cool for 30 minutes under conditions identical with those used for treatment of C. fascians (Procedure 1). The solution was neutralized with 0.1 NH_4OH and the reaction mixture was chromatographed on a Celite column (1.9 cm diameter x 50 cm length). The first 15 ml of eluate was collected and the contents were chromatographed on Whatman 1 paper in solvent B together with authentic i^6Ade. A single area of radioactivity was found on the chromatogram and coincided with the mobility of i^6Ade (Figure 33). Since this technique does not give specific labelling of the i^6Ado residues in tRNA I could not quantitate the result, but it shows that i^6Ade is liberated from the tRNA of C. fascians under these extraction conditions.

Bioassay of the cytokinin activity in C. fascians medium

It appeared that a considerable quantity of i^6Ade was released from C. fascians tRNA by heating and acidification. Klambt et al. (1966) reported that other cytokinin activities were present in extracts of

FIGURE 34

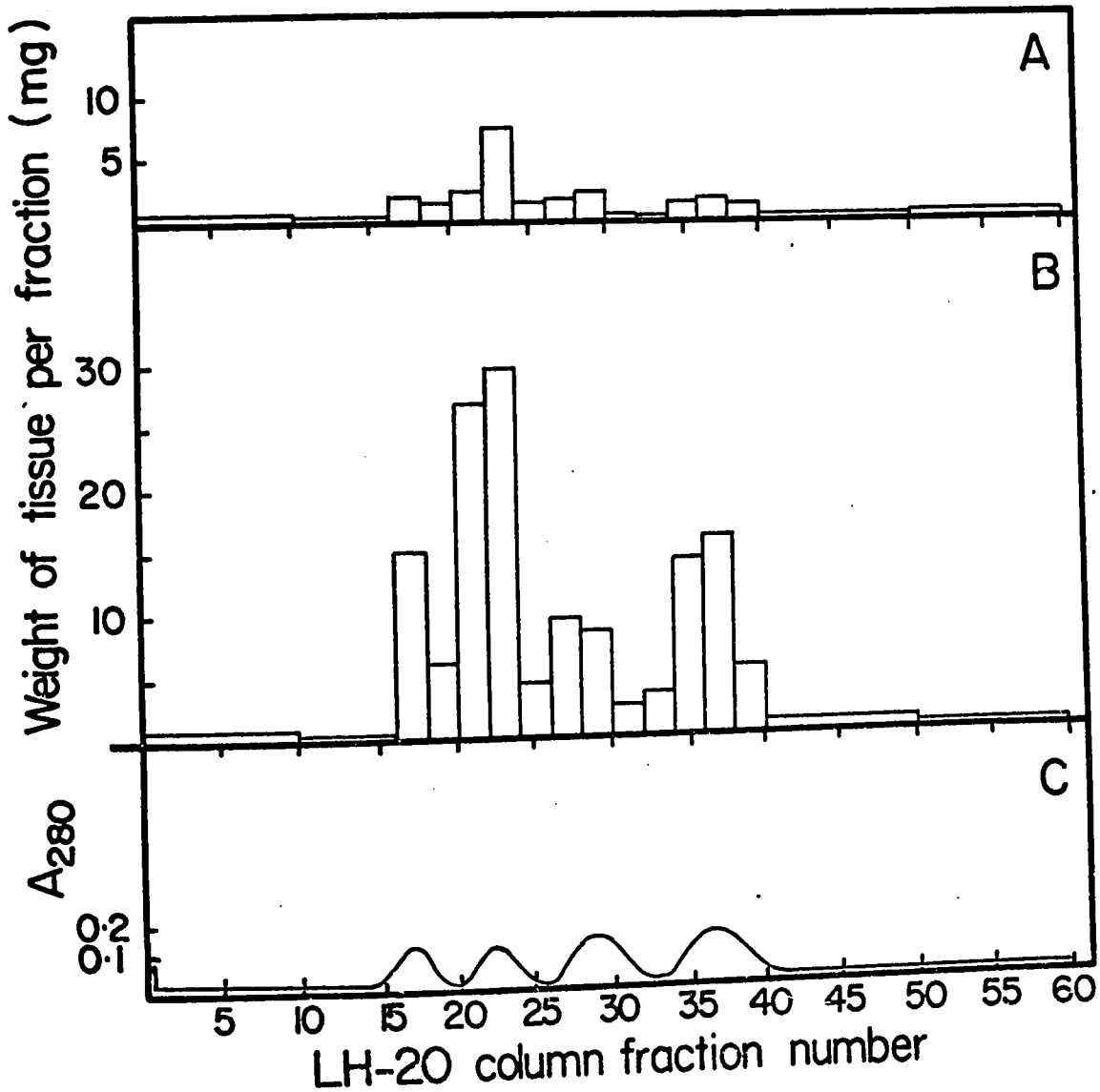
BIOASSAY OF CYTOKININ ACTIVITY FROM CULTURES OF C. FASCIANS

A C. fascians culture (4 litres) was extracted as described on page 60. Fractions (12 ml) from the LH-20 column were bioassayed as described on page 61. Fractions 1 to 10 were pooled, as were fractions 11 to 16, 41 to 50, and 51 to 60. Fractions 17 to 40 were assayed in pairs. From each fraction 0.1 ml, 1.0 ml and 4.9 ml samples were bioassayed. Each bioassay was done in duplicate. After 35 days the tissue from each flask was weighed. The mean weight of tissue in each pair of bioassays is represented in a histogram against the corresponding LH-20 column fraction.

A. 0.1 ml from each fraction per bioassay.

B. 1.0 ml from each fraction per bioassay.

C. The ultraviolet absorption profile of a separation of four standard cytokinin compounds: zeatin, ribosyl zeatin, 1^6Ade and i^6Ado on the same LH-20 column.


A
B
C
Weight of tissue per fraction (mg)
10
5
30
20
10
A_{280}
0·2
0·1
5 10 15 20 25 30 35 40 45 50 55 60
LH-20 column fraction number

C. fascians medium. Using the solvent extraction procedure the i^6Ade formed by hydrolysis of tRNA would not contribute to the cytokinin activity of the cultures. In that case the contribution of the other cytokinin activities would be much greater. Therefore, C. fascians cultures were extracted by Procedure 2 (page 155) and their cytokinin activity determined using a bioassay procedure.

A culture of C. fascians (4 litres) was treated by extraction Procedure 2 (no heating or acidification). The LH-20 column eluate was divided into 12 ml fractions. Aliquots of 0.1 ml and 1 ml were taken from each fraction for the assay. The aliquots of fractions 1-10, 11-16, 41-50 and 51-60 were pooled for the bioassay. Other fractions (17-40) were assayed in groups of two.

The bioassays were done by the method of Dyson et al. (1970), described on page 61. The results are shown in Figure 34A,B and are superimposed over the LH-20 column profile of four authentic cytokinin compounds: zeatin, ribosyl zeatin, i^6Ado and i^6Ade (Figure 34C).

Cytokinin activity was present in four distinct parts of the eluate from LH-20 column. Approximately 20% of the total activity chromatographed coincidentally with i^6Ade. The majority of the cytokinin activity chromatographed coincidentally with zeatin. Lesser amounts of cytokinin activity eluted from the LH-20 column in the same fractions as i^6Ado and ribosyl zeatin.

This experiment was done twice, with exactly the same results on both occasions. Bioassays were done using various amounts of each fraction to ensure that any growth response observed was not masked by toxic materials present in the extract.

It is not possible to determine the exact quantity of cytokinin present by bioassay techniques. However, the amount of growth stimulation that was produced by 1 μM N^6-(benzyl)adenine, was approximately 5 to 10 times that produced by 1 ml of each of LH-20 column fractions 35 and 36 or 37 and 38.

Therefore, I conclude that the cytokinin activity due to i^6Ade in cultures of C. fascians is compatible with the small quantities estimated to be present by gas chromatographic and radioisotopic methods described previously.

DISCUSSION

DISCUSSION

The Discussion is in four parts:

(i) The metabolism and biological effects of i^6Ado on 6410 cells.

(ii) The effects of i^6Ado on tobacco pith tissue.

(iii) The origin of free i^6Ade.

(iv) A general discussion of the work.

(i) The metabolism and biological effects of i^6Ado

N^6-(Δ^2-Isopentenyl)adenosine is a natural component of the tRNA of all organisms so far investigated (Hall, 1971). In the tRNA molecules that contain i^6Ado, it is located adjacent to the anticodon. N^6-(Δ^2-Isopentenyl)adenosine plays an important role in ensuring correct codon-anticodon interactions (Gefter and Russell, 1969; Fittler and Hall, 1966). Free i^6Ado is a potent cytokinin, initiating cell division and differentiation in certain plant tissues (Skoog *et al.*, 1967). Because of the widespread distribution of i^6Ado in animals, plants and micro-organisms, and because this compound causes differentiation in certain tissues, its effects on various mammalian cells - normal and neoplastic - were tested (Grace *et al.*, 1967). These workers found that i^6Ado inhibited *in vitro* Sarcoma S-180, and 6410 cells derived from the blood of a patient with chronic myeloid leukaemia. As a result of these experiments, i^6Ado was given a therapeutic trial in patients with leukaemia and related neoplasms. At this time, and against this background, the work on 6410 cells described in this thesis was done.

The 6410 cell line used in these studies was derived from a patient with chronic myeloid leukaemia (Iwakata and Grace, 1964). At that time, and in later publications, 6410 cells have been described as "myeloblastic" (Grace et al., 1967; Fleysher et al., 1969). I found that cells of this line had predominantly a normal number of chromosomes, forty-six. However, many of the chromosomes were abnormal (p.63). The abnormalities were heterogenous and consisted predominantly of loss of pieces of chromosomes that had rearranged and attached themselves to other chromosomes. In acute leukaemia, many chromosomal abnormalities have been reported (Ford et al., 1958; Jacobs et al., 1959; Baikie et al., 1959; Kinlough and Robson, 1961). Such chromosomal abnormalities are diverse and their significance is undetermined. In chronic myeloid leukaemia, however, a much more constant abnormality has been found. A small acrocentric chromosome replaces chromosome 21, one of the smallest acrocentric chromosomes. This Philadelphia, or Ph^1 chromosome occurs in over 90% of cases of chronic myeloid leukaemia (Nowell and Hungerford, 1960, Baikie et al., 1960; Fitzgerald et al., 1963). It has been found in both treated and untreated cases, and in preparations from both peripheral blood and bone marrow (Adams et al., 1961; Kinlough and Robson, 1961). I found no evidence of the presence of Ph^1 chromosome in 6410 cells. This suggested that these were not the typical cell of chronic myeloid leukaemia. Further, the abnormal chromosomal morphology, that varied from cell to cell, indicates that these cells have altered considerably since they were first isolated. It is possible that because of these chromosomal changes the physiological characteristics of the cell line have also altered. Subsequent to completion of this

work I have learned that these cells almost certainly do not represent the cell line of the disease, and are better considered as a line derived from immature haemapoietic cells (Dr. George Moore, personal communication, 1971). Furthermore, it is general experience that lymphoid cell lines are more commonly established from cultures of peripheral blood than are myeloid cell lines. Although this in no way invalidates the results obtained in this study, it does mean that the results cannot necessarily be applied to the *in vivo* situation, and the true cell of the disease in chronic myeloid leukaemia.

This cell line grew *in vitro* in medium RPMI 1629 containing 15% foetal calf serum. The cells did not grow when the initial cell concentration of the cultures was as low as 0.5×10^5 cells per ml (p.64). This result contrasts with that of other workers (Fleysher *et al.*,1969) who were able to culture 6410 cells at this low concentration. There are two likely reasons for the differences between my findings and those of Fleysher *et al.* (1969). Firstly, the cell line may have changed its characteristics during subculturing. Certainly the chromosomal abnormalities in these cells suggest that they had changed since they were originally isolated. The second possibility is that the serum used in the culture medium did not contain the same stimulatory factors as that used by other workers. The growth stimulating activity of serum varies considerably from sample to sample and some samples of serum could exert quantitatively and qualitatively different effects on these cells in culture. The fact that I obtained essentially similar results over a period of time using different batches of serum, suggests that the different growth characteristics that I obtained from those of Fleysher *et al.* (1969) were not due to this factor.

Because i^6Ado stimulates the growth of certain plant tissues *in vitro* and *in vivo* (Hall and Srivastava, 1968; Dyson and Hall, in press) I investigated whether this compound had any stimulatory effects on the growth of 6410 cells cultured *in vitro*. I found no evidence that i^6Ado had growth stimulatory effects at the concentrations used (Fig.7,8). This contrasts with the results of Fleysher *et al*. (1969). These workers found that i^6Ado and some other N^6-substituted adenosine compounds: N^6-(furfuryl)adenosine, N^6-(phenyl)adenosine, N^6-(2-ethoxy-ethyl)adenosine, N^6-(allyl)adenosine, N^6-(propyl)adenosine and N^6-(isopropyl)adenosine in low concentrations stimulated the growth of 6410 cells *in vitro*. They did not report whether the effect of i^6Ado was to increase the rate of cell division in logarithmic growth phase, or to reduce the period of the lag phase. Fleysher *et al*. (1969) only reported the initial and final cell concentrations in the cultures, whereas in the experiments reported in this thesis these values were determined at frequent intervals through the growth period, and therefore would have detected a difference in the length of the lag period. The initial cell concentration used by Fleysher *et al*. was 0.5×10^5 cells per ml, a concentration considerably lower than that used in my work. At such low concentrations cell cultures have a long lag phase. Therefore, any compound that could shorten the duration of this would cause an increase in the number of cells present in the cultures at a given time, without actually affecting the growth rate of the cells. Fleysher *et al*. (1969) diluted the cultures during the course of the experiment. This dilution would introduce a second lag phase and therefore accentuate any difference in the cell concentrations at the end of the experiment.

An alternative explanation of the differences in results is that the cell line may have changed during subculture, as discussed earlier. In this context it must be noted that other cell lines treated with i^6Ado including Sarcoma S-180, LKID, HRIK, and RPMI 5287 were not stimulated (Fleysher et al., 1969). N^6-(Δ^2-Isopentenyl)adenosine stimulates two other animal systems in vitro. Gallo et al. (1969) have reported that i^6Ado stimulates DNA synthesis and mitosis in circulating blood lymphocytes that have been transformed by phytohaemagglutinin in vitro. Neurite outgrowth from embryonic chick sympathetic ganglia cultured in vitro is also stimulated by low concentrations of i^6Ado in a manner similar to that seen when these cells are treated with Nerve Growth Factor (Rathbone and Hall, unpublished observations). Despite these cases, it appears that stimulation of animal cells in vitro by i^6Ado is relatively uncommon, compared with the frequency of this phenomenon in plant tissues. There have been no reports that i^6Ado stimulates the growth of animal cells in vitro, although Jones et al. (1968) reported that in patients with chronic myeloid leukaemia, treatment with i^6Ado increased either the production or the release of blast cells when these were the predominant cell, whereas when the promyelocyte was predominant, i^6Ado therapy led to remission. However, Suk et al. (1970) in experiments in rats, demonstrated that the granulocytosis that followed the administration of i^6Ado was due to the release of mature granulocytes from the bone marrow, and that the rates of proliferation and differentiation of the haematopoietic cells were unaffected. Therefore it is probable that the leucocytosis observed by Jones et al. (1968) was due to the release of blast cells rather than

stimulation of their production. Because of the relative infrequency with which i^6Ado stimulates the growth of animal cells either *in vivo* or *in vitro* any change in the genetic properties of 6410 cells would be more likely to change them from a line that was stimulated by i^6Ado to one that was not. Therefore, I favour this as an explanation of the differences in the results reported by Fleysher *et al.* (1969) and those reported in this thesis.

N^6-(Δ^2-Isopentenyl)adenosine was toxic to 6410 cells at concentrations of 3 μM or more (Fig. 7,8). The cell viability decreased within 2 hours of the addition of 3 μM i^6Ado to the cultures and, within 30 hours, the ratio of live to dead cells was 0.4. Although in agreement with the results of other workers (Grace *et al.*, 1967; Fleysher *et al.*, 1969) that i^6Ado was toxic to these cells, I found it to be more toxic than reported elsewhere. For example, Fleysher *et al.* (1969) report that 9 μM i^6Ado caused only a 50% decrease in viability after 48 hours and that even after 5 days growth in the presence of 29 μM i^6Ado the total cell concentration was 5.9×10^5 cells per ml compared with 6.4×10^5 cells per ml in the control cultures. 6410 cells grew normally in media that contained 2 μM i^6Ado, a concentration just below that toxic to 6410 cells. Therefore I conclude that there is a definite threshold effect in the toxicity of i^6Ado to these cells. The reasons for the toxicity of i^6Ado and the threshold effect will be considered later when the metabolism of i^6Ado by these cells is discussed.

Although i^6Ado did not stimulate the growth of 6410 cells, it was felt that a sensitive indicator might show that i^6Ado caused a maturation of these cells. Alkaline phosphatase activity was chosen as

an indicator because leucocyte alkaline phosphatase is usually reduced or absent in chronic myeloid leukaemia, but returns to normal after remissions or chemotherapy (Wachstein, 1946; Valentine et al., 1957; Xefteris et al., 1961). No change in the alkaline phosphatase activity of these cells was observed when they were cultured in the presence of i^6Ado (Fig.9). However, as it is now known that these cells probably do not represent the cell of the disease, then this experiment should be repeated using fresh cells from patients treated with i^6Ado. Under these circumstances the composition of the cell population under investigation would be known.

Blakeslee (personal communication, 1968), using histochemical techniques, found an increase in the lactic dehydrogenase (LDH) activity of 6410 cells that had been treated with i^6Ado. Using biochemical estimations of LDH activity I found no alteration in LDH activity per cell following treatment with i^6Ado at concentrations that were not toxic to the cells (Fig.10). However, I found that LDH activity increased as the numbers of dying cells in the population increased and was independent of the presence of i^6Ado. Slocum et al. (1972) have recently proposed that the toxicity of i^6Ado to Sarcoma S-180 in vitro is due to the formation of the corresponding 5' monophosphate (i^6AMP), that inhibits adenylate kinase. Inhibition of adenylate kinase causes a depletion of the ATP in the cells, and consequently their death. It is reasonable that LDH might be induced in such circumstances. However, the induction of LDH was not specific to i^6Ado treatment.

Having established the toxicity of i^6Ado to 6410 cells, I questioned whether 6410 cells contained this potentially toxic material,

or a derivative, as do all other cells that have been investigated (Hall, 1971). As has been discussed earlier (page 13) i^6Ado is important for correct tRNA-codon interaction (Gefter and Russell, 1969; Fittler and Hall, 1966). Using radioactive labelling techniques it was demonstrated that 6410 cells contained i^6Ado in their tRNA but not in higher molecular weight species of RNA (p.89). The presence of i^6Ado has not been reported in species of RNA other than tRNA (Hall, 1971).

The quantity of i^6Ado present was estimated on the basis of the quantity of [8 -^{14}C] i^6Ado present compared with the total label incorporated into the tRNA. N^6-(Δ^2-Isopentenyl)adenosine comprises approximately 0.03 moles per cent of the total nucleosides in 6410 cell tRNA. This value is in close agreement with the values reported by others in other systems (Hall, 1971). These results do not rule out an abnormality in the metabolism of i^6Ado in the tRNA of these cells. However, it is unlikely that the toxic effects of i^6Ado on 6410 cells is related to the presence of a grossly abnormal amount of i^6Ado in their tRNA.

There is evidence for turnover of tRNA in intact organisms (Agarwal and Weinstein, 1970; Hanoune and Agarwal, 1970). Furthermore, characteristic components of tRNA have been found in the urine of humans and other animals (Chheda, 1970). Therefore, it is probable that due to tRNA turnover, 6410 cells are also exposed to the degradation products of tRNA, including i^6Ado. Therefore, I examined the cells and the culture medium for the presence of i^6Ado, and two of its possible metabolites, i^6Ade and i^6AMP. I found no evidence of the presence of either metabolite in the cells or in the culture medium (p.80, 87). The limits

of detection of this procedure may be judged from essentially similar experiments on Corynebacterium fascians reported elsewhere in this thesis. In those experiments (page 135) i^6Ade was detected at concentrations as low as 1.5 nM. The significance of this negative finding will be considered further when the degradation of i^6Ado by 6410 cells is discussed.

Having established the presence of i^6Ade in the tRNA of 6410 cells I investigated its biosynthesis under normal conditions and in tRNA from cells that had been grown in the presence of i^6Ado to determine whether exogenous i^6Ado inhibited the biosynthesis of i^6Ado *in vivo*. 6410 cells contain an enzyme activity that converts [2 -^{14}C] mevalonic acid to i^6Ado on preformed *E. coli* tRNA molecules(p.91). *E. coli* tRNA was used as a substrate because it has been demonstrated that yeast and mammalian sources contain an enzyme activity capable of isopentenylating this tRNA, in excess of the isopentenylation performed by the *E. coli* enzymes *in vivo* (Kline *et al.*, 1969). This enzyme system is analogous to that isolated from other sources (Fittler *et al.*, 1968b; Kline *et al.*, 1969; Chen and Hall, 1969). Kline *et al.* (1969) have demonstrated that Δ^2-isopentenyl groups are removed from tRNA molecules by treatment with potassium permanganate. This Δ^2-isopentenyl deficient tRNA will serve as a substrate for Δ^2-isopentenyl pyrophosphate: tRNA-Δ^2-isopentenyl transferase. If 6410 cell tRNA did not have a full complement of Δ^2-isopentenyl groups it would therefore be expected to act as a substrate for Δ^2-isopentenyl pyrophosphate: tRNA-Δ^2-isopentenyl transferase from 6410 cells. However, when 6410 cell tRNA was incubated with an enzyme system from the same cells, no additional Δ^2-isopentenyl

groups were transferred to the tRNA. Therefore, I concluded that 6410 cell tRNA was not deficient in Δ^2-isopentenyl groups (Table 4).

The possibility that free exogenous i^6Ado would suppress the synthesis of this compound in the tRNA was investigated by similar experiments in which the tRNA was extracted from cells that had been cultured in the presence of the highest concentration of i^6Ado that did not affect their viability (Table 4). There was no evidence that this tRNA lacked Δ^2-isopentenyl groups. Therefore, I conclude that the presence of free i^6Ado does not control the synthesis of this compound in the tRNA of these cells.

6410 cells apparently synthesize normal amounts of i^6Ado in their tRNA. The tRNA of rat liver has a half life of 5 days (Hanoune and Agarwal, 1970) and in humans, modified components of the tRNA are found in the urine (Chheda, 1970), suggesting a turnover of tRNA. Such a process would expose 6410 cells to a compound that is toxic to them in micromolar concentrations. A detoxification mechanism would enable the cells to deal with this compound. In human and other bone marrow there is a strong enzymic activity that catalyzes the degradation of i^6Ado. Since no free i^6Ado was found in 6410 cells or in their growth medium this was further evidence for the presence of a similar mechanism in 6410 cells.

I found an enzyme activity in 6410 cells that converted i^6Ado to the corresponding base i^6Ade(p. 98). The base is 50 times less toxic to 6410 cells than i^6Ado (Fleysher et al., 1969; Jones et al., 1968). This activity therefore represents a mechanism by which i^6Ado is detoxified by these cells. There was no evidence of any activity in these cells

that cleaved the N^6-(Δ^2-isopentenyl) sidechain from i^6Ado. This is in contrast to a strong adenosine aminohydrolase activity present in human and other bone marrow that converts i^6Ado to inosine (Hall and Mintsioulis, 1972; Hall et al., 1971).

The significance of a pathway of degradation of i^6Ado in 6410 cells, which is different from that in the cells of normal bone marrow, is unknown. However, it may relate to a different physiological role of i^6Ado and its derivatives in the different cells, and also to its quantitatively different toxic effects on various types of cells. Dyson and Fox have postulated that the effects of cytokinins on plant cells are directly related to their metabolism in those tissues (Dyson et al., 1972). The same may also be true in animal systems.

Free i^6Ade was not detected in 6410 cells or their culture medium after 6410 cell cultures had been labelled with radioactive adenosine(p.80,87). Because concentrations of these compounds as low as 1.5 nM would have been detected by the methods used, I conclude that only very small quantities of these compounds are normally present in 6410 cells. In vitro, i^6Ade was the only metabolite of i^6Ado. Due to tRNA turnover releasing i^6Ado, i^6Ade should have been detected if this was the sole product of degradation of i^6Ado in vivo. Therefore, I conclude that either the turnover of tRNA is slow under the conditions of the experiment, or that i^6Ade is metabolized further by enzymes that have not yet been detected in 6410 cells, or that i^6Ado is also metabolized by other pathways in these cells in vivo.

In Sarcoma S-180 cells i^6Ado is metabolized rapidly (Slocum et al, 1972). Within 15 minutes they demonstrated over 80% of [8 -^{14}C]

i^6Ado is converted to i^6AMP. N^6-(Δ^2-Isopentenyl)adenosine 5' monophosphate was toxic to the cells because it inhibited adenylate kinase, and resulted in a shortage of ATP in the cells. I found no radioactive i^6AMP in 6410 cells or their medium following incubation with [8 - ^{14}C] adenosine(p.87). Under physiological conditions, therefore, i^6AMP is not synthesized in large quantities in these cells, although such a reaction may be readily demonstrable in S-180 cells that have been cultured with [8 - ^{14}C] i^6Ado (Slocum et al., 1972)in vitro. Divekar and Hakala (1971) have shown that the toxicity of N^6-(substituted) adenosine analogues is related to their ability to serve as substrates for adenosine kinase, at least in sarcoma S-180 cells. A similar mechanism may operate in 6410 cells. If this is so, then the threshold effect of i^6Ado toxicity in 6410 cells may represent the saturation of the enzyme system that degrades the small, physiological amounts of i^6Ado to which 6410 cells are exposed during tRNA turnover. This would allow adenosine kinase to phosphorylate the excess i^6Ado.

Because neither i^6Ade nor i^6AMP were found in these cells it is also possible that these compounds are metabolized further, although more work is necessary before a definitive answer to this point can be made.

(ii) The effects of i^6Ado on tobacco pith tissue

N^6-(Δ^2-Isopentenyl)adenosine has predominantly inhibitory effects on RPMI 6410 cells. No stimulatory or differentiation-inducing effects were observed in this system, and no free i^6Ado or i^6Ade was detected in these cells. However, because all sources investigated contain i^6Ado in their tRNA (Hall, 1971), and because of the role of free i^6Ado or a related compound in growth and differentiation of plant tissues, it

appeared possible that this nucleoside might have a similar regulatory role in animal tissues. A knowledge of the biological effects and metabolism of this nucleoside in plant systems might therefore indicate fruitful directions of future research into possible analogous positive biological actions of this nucleoside in animals.

Studies have been made of the effects of cytokinins on tobacco pith tissue in culture (Skoog and Armstrong, 1970). Recently the energy metabolism, and in particular the starch metabolism of the tissue has been suggested to be of prime importance in the cytokinin-induced differentiation of tobacco pith callus (Thorpe and Murashige, 1970). They found that gibberellin, a plant hormone that prevented an increase in starch content of tissue by inducing α-amylase, inhibited cytokinin-induced organ formation. Nitsche (1968) has shown that gibberellin acts synergistically with cytokinins in causing cytokinesis. Therefore, there appear to be two different cytokinin-induced mechanisms. One, cytokinesis, that does not require the hydrolysis of large stores of starch and secondly, organogenesis, that does require the hydrolysis of large stores of starch.

To investigate this point I used a variant strain of tobacco pith tissue that had arisen spontaneously from normal KX-4 tissue in our laboratory. The variant KX-4/R1 required cytokinin for growth, but did not produce mature organs when it was treated with high concentrations of cytokinins (3 μM or greater).

The growth response to i^6Ado of the KX-4/R1 variant and normal KX-4 tissue were compared (Fig.20,28). The fresh weight of the KX-4 tissue that had been grown in the presence of i^6Ado was greater than that of the control

tissue. Growth varied with the concentration of i^6Ado that was used, 0.6 μM causing the greatest increase in fresh weight. At concentrations of i^6Ado above 0.6 μM the growth of the tissue was inhibited, but the tissue differentiated. In contrast to this response, the KX-4/R1 tissue was stimulated maximally by a much higher concentration of i^6Ado, 3 μM and no pronounced inhibition of the gain in fresh weight was observed even in the presence of 9 μM i^6Ado.

The fresh weight of the tissue is composed largely of water. The dry weight is made up of the cell walls and cell constituents other than water. It is not surprising, therefore, that the more differentiated organized tissues - KX-3 tissue that had been treated with 3 μM i^6Ado or 6 μM i^6Ado - had a greater proportion of dry weight than the relatively unorganized tissue that was starved of cytokinins.

The dry weight of the KX-4/R1 variant was 5.5% of the fresh weight compared with the normal KX-4 tissue that had a dry to fresh weight ratio of 9.5%(Fig.20,28). This reflects the greater degree of organization of the latter tissue, even in the absence of i^6Ado. The difference in the ratios of dry to fresh weights in the two types of tissue was most marked at high concentrations of cytokinins. N^6-(Δ^2-Isopentenyl)adenosine at concentrations of 3 μM or more strongly inhibited the growth of KX-4 tissue, but concentrations of i^6Ado as high as 9 μM only weakly inhibited the growth of KX-4/R1. The reverse effect was noted with respect to the dry to fresh weight ratio of the tissues. When treated with 6 μM i^6Ado, 25% of the KX-4 tissue was dry weight, but only 2% of the KX-4/R1 tissue was dry weight even when treated with 9 μM i^6Ado.

As discussed above, more ordered tissues have a greater

concentration of material in them than do less organized ones. Therefore I conclude that the effect of high concentrations of i^6Ado on KX-4 tissue is to increase its order, whereas the order of KX-4/R1 tissue is not increased by treatment with cytokinins.

Histological studies of KX-4/R1 tissue showed that, although it was unable to complete differentiation, the tissue did form rudimentary areas of rapidly dividing cells - Torrey's meristemoids (Fig.26) - that are characteristic of plant tissues that are undergoing differentiation (Torrey, 1966). The initial changes observed in the tissues were typical of those seen in tobacco callus tissues that are differentiating (Thorpe and Murashige, 1970).

A striking reduction in cell diameter occurred within 48 hours following administration of i^6Ado to the KX-4/R1 tissue(Fig.27). The reduction in average cell diameter to approximately 60% of that prior to cytokinin treatment could be accounted for if each cell had undergone one cell division without significant growth. Thus, the initial cytokinin action - cytokinesis - occurrs within the first few hours after the administration of the cytokinin in order that at least one cycle of cell division might be completed within 48 hours. The mean cell diameter remained essentially constant throughout the experiment after the initial decrease indicating that the cells were dividing and maintaining a constant cell diameter that was lower than was possible in KX-4/R1 tissue before the addition of i^6Ado.

The starch content in KX-4/R1 tissue increased throughout the 24 days of the experiment (Fig.27). Cells that had a small diameter contained more starch granules than cells with a larger diameter. The small meristemoid cells contained the most starch. My interpretation of these

results is at variance with that of Thorpe and Murashige (1970) who found that there was no starch accumulation in non-organ-forming tobacco tissue. These workers reported that in tissue that formed organs there was an extensive starch accumulation, that was followed by its rapid hydrolysis immediately before the formation of meristemoids. They interpreted these results as showing that meristemoid formation, that is the initial stage of morphological differentiation, was dependent on the accumulation of starch and upon its subsequent hydrolysis. Clearly, my results show that the formation of meristemoids does not depend on the hydrolysis of starch reserves. The hydrolysis of starch may be required for a later stage in organogenesis of tobacco pith tissue. This would be in agreement with the findings of Thorpe and Murashige (1970) and of Marinos (1967) who found that the starch concentration was low in the young leaves and shoot apex of the developing potato, presumably because it had been hydrolysed during differentiation, whereas the starch content of the underlying parenchyma was high. These results show that in addition to starch accumulation, starch hydrolysis is necessary for the completion of organogenesis in tobacco pith tissue. However, the initial stage of organogenesis, the formation of Torrey's meristemoids, does not require the hydrolysis of starch as was previously believed (Thorpe and Murashige, 1970).

(iii) The origin of free i^6Ade

The biosynthesis of cytokinins is an important aspect of their metabolism. The results of experiments using tobacco pith tissue *in vitro* have shown that the concentration of cytokinins, and in particular the ratio of cytokinin to auxin in the culture medium of plant tissues,

is important in determining the growth response of the tissue. These findings confirm the results of Skoog and Miller (1957) who were the first to report this phenomenon. The amount of cytokinins in any tissue is regulated by rates of their synthesis and degradation. Thus, the mechanism(s) and control of cytokinin biosynthesis play a crucial role in the differentiation of plant tissues.

Initial studies on i^6Ado biosynthesis were done using a variant strain of KX-4 tobacco pith tissue that synthesizes cytokinins. Because of the small quantities of i^6Ado that are synthesized by this tissue (Dyson and Hall, 1972) this system was not sensitive enough to use in this type of study. The study of the mechanism of cytokinin biosynthesis was therefore done using a plant pathogen, Corynebacterium fascians. This organism causes a fasciation disease in plants, due to manufacture and subsequent release of cytokinins into the plant (Thimann and Sachs, 1966). Klambt et al.(1966) have isolated relatively large amounts (10 μg per litre) of i^6Ade from cultures of this bacterium. They estimated that this cytokinin was present in the cultures in concentrations of at least 100 μg per litre.

Initial experiments showed that these bacteria took up from their medium, into tRNA, radioactive adenine, but not mevalonic acid(Fig.33&p.142). Therefore, radioactive mevalonic acid could not be used as a specific precursor to label the sidechain of i^6Ade. [8 -^{14}C] Adenine was incorporated into the i^6Ade synthesized by the bacteria(Fig.30). No difference was observed in the amount of label incorporated into the i^6Ade when the cultures were labelled with divided doses of [8 -^{14}C] adenine throughout the growth phase, or with the same quantity of [8 -^{14}C]

adenine given as a single pulse to the bacteria when they were in late logarithmic growth phase (Fig.31). This is surprising in view of the report that cultures of C. fascians harvested in stationary phase contained more i^6Ade than those harvested earlier (Klambt et al., 1966). In each experiment I determined the recovery of i^6Ade by the addition of unlabelled i^6Ade to the medium before the extraction procedure. The recovery remained fairly constant in the range of 25% to 35% for all extractions.

The quantities of [8 -^{14}C] i^6Ade recovered from cultures of C. fascians were smaller than would have been expected on the basis of the uptake of [8 -^{14}C] adenine by the bacteria and from the estimate made by Klambt et al. (1966) of the quantity of i^6Ade present in these cultures. This caused me to question the quantity of i^6Ade present in cultures of C. fascians. Thimann and Sachs (1966) extracted considerably less cytokinin activity from C. fascians than did Klambt et al. (1966). However, Thimann and Sachs used an oat leaf bioassay system (Gunning and Barkley, 1963) to determine the amount of cytokinin activity whereas the latter group used the tobacco pith bioassay system (Rogozinska et al., 1964). These systems respond differently to different cytokinins: the tobacco pith bioassay is relatively more sensitive to i^6Ade and related compounds (Rogozinska et al., 1964) than oat leaf bioassay. This difference in sensitivity of the bioassays would explain some of the difference between the estimates of cytokinin activity present in the cultures. Klambt et al. (1966) further proposed that the extraction procedure that they had used was more efficient than that used by Thimann and Sachs (1966).

I estimated the quantity of i^6Ade in the cultures by extracting the cultures with solvent, chromatographing the extract on a Celite-545 partition column and then on a column of LH-20, developed with 35% ethanol. As a final step, the putative i^6Ade was chromatographed on a gas:liquid chromatogram (GLC)(Fig.32). The amount of i^6Ade obtained by this technique was so low that it was at the threshold of detectability. The relatively small amount of i^6Ade isolated made it difficult to separate it completely from the background. However, by comparing areas under the peaks that corresponded to i^6Ade that was run as a control, 0.5 µg to 1 µg of this compound were estimated to have been extracted from each litre of culture medium. Assuming that the recovery of i^6Ade after the extraction procedure was 25% to 35%, then the quantity of i^6Ade originally present in the cultures was no more than 1 µg to 4 µg per litre.

There was a significant difference between the extraction procedures used by Klambt et al. (1966) and Thimann and Sachs (1966). The former included heating and mild acid treatment in their extraction procedure. However, Thimann and Sachs used a solvent extraction similar to the one used in the experiments reported in this thesis. Because the solvent extraction procedure resulted in a final yield of 25% to 35% of the i^6Ade present in the cultures, it seemed that the procedure used by Klambt et al. (1966) released i^6Ade from sites that were not accessible using other techniques.

To clarify this point, a culture of C. fascians was divided into two aliquots. The first was extracted using the author's own solvent extraction procedure; the other was heated and subjected to a

mild acidification step as described by Klambt et al. (1966) before it was extracted in exactly the same manner as the other half of the culture. Identification of i^6Ade was made on the basis of its known chromatographic behaviour on partition chromatography on Celite-545, chromatography on Sephadex LH-20, and finally, by its behaviour on gas:liquid chromatography (GLC). It should be noted that the GLC technique separates i^6Ade from other known cytokinins. The compound isolated from the extract cochromatographed in the GLC with i^6Ade.

The amount of i^6Ade in the extract obtained by the solvent extraction procedure was small and at the threshold of detectability. The relatively small amount of i^6Ade that was isolated made it difficult to separate completely from background. In addition, compounds of this type chromatograph early in this GLC system and are therefore seen as a shoulder on the solvent peak. Thus, any estimate of quantities of material present could have as much as two-fold error. It was estimated that approximately 0.5 µg per litre to 1 µg per litre of i^6Ade were extracted per litre of culture using solvent extraction. Assuming an efficiency of extraction of 25% to 35%, i^6Ade was present in the original cultures in concentrations between 1.0 µg per litre and 4 µg per litre. The portion of the culture that was extracted by the procedure that included heating and mild acidification yielded a much greater quantity of i^6Ade (Fig.32). This was estimated to be 10 µg to 12 µg per litre. This suggests that approximately 36 µg to 48 µg per litre of i^6Ade were present in the culture following the heating and acidification steps.

Because of the inherent errors in estimating the exact quantities of material present against a high sloping background of GLC, another type of experiment was done. A culture of C. fascians in which the i^6Ade content was labelled with a radioactive marker was analyzed(p.159). This culture was divided in half and the free (unbound) i^6Ade isolated by identical procedures except that one included heating and mild acidification. The latter procedure yielded 5.5 times more i^6Ade than was found when the solvent extraction procedure was used solely. The i^6Ade was identified as previously described by means of its behaviour in the extraction procedure, by partition chromatography on Celite-545 and chromatography on Sephadex LH-20 columns and by its chromatographic behaviour on paper chromatography.

The five and a half fold difference obtained in the experiment using C. fascians in which the i^6Ade had been labelled with radioactive adenine represents a considerably more accurate measure of the difference between the two extraction procedures than can be obtained using gas chromatography. However, data using the radioactive material do not furnish absolute values as do the gas chromatography data. The value of 12 μg per litre of i^6Ade yielded by cultures treated by the hot acidification step and measured by GLC, is a relatively accurate estimate. The figure of 0.5 μg to 1 μg per litre of i^6Ade yielded by the procedure using only solvent extraction gives only an estimate, although the value is certainly considerably less than that extracted when the cultures are heated and acidified. A more accurate value for the yield of i^6Ade by the procedure using only solvent extraction can be made using the results of the radioactive experiment discussed above and the relatively

accurate absolute value of 12 μg of i^6Ade per litre obtained by GLC for the procedure that included heating and acidification. I calculate that, allowing for losses during the extraction procedure, approximately 5.0 μg per litre were present in the C. fascians cultures at the beginning of the extraction procedure, that had not been treated with heating and acidification. The estimates obtained using both GLC data and radioactivity data are in surprisingly good agreement with those estimated using GLC alone, that are about one half of this value.

The source of the considerably greater quantity of i^6Ade extracted by the heating:mild acidification step could be the result of liberation of i^6Ade from a bound form. There are at least two possibilities. Firstly, i^6Ade may be bound to a protein from which the heating and acidification treatment extract it more efficiently than the solvent extraction procedure. If this were the case, it is reasonable to assume that other cytokinins produced by C. fascians would also be bound in a similar manner and heating and acidification would liberate all the cytokinins more effectively. Klambt et al. (1966) reported that after their extraction procedure - that included heating and acidification - i^6Ade represented by far the predominant cytokinin activity in the extract. On the other hand, when the heating and acidification steps were omitted I found that i^6Ade accounted for only a small portion of the total cytokinin activity(Fig.34). Thus, i^6Ade is apparently extracted preferentially by the heating:acidification treatment. Therefore, it is doubtful that the additional amount of i^6Ade extracted after heating:acidification can be attributed to a protein bound form of cytokinin in the cultures.

The second possibility is that the source of the greater quantity of i^6Ade extracted using heating:acidification procedures is from the

tRNA. Matsubara et al. (1968) showed that hydrolysates of C. fascians tRNA contain cytokinin activity. On the basis of chromatographic mobility they suggest that the cytokinin is i^6Ade.

Since Klambt et al. initially reported the presence of i^6Ade in C. fascians cultures, new information about the occurrence as well as the chemical properties of i^6Ade and its related compounds has become available. Matsubara et al. (1968) report that only one cytokinin, tentatively identified as N^6-(Δ^2-isopentenyl)adenosine, is present in the tRNA of C. fascians. The work reported in this thesis confirms the presence of i^6Ado in C. fascians tRNA. Mild acid treatment of tRNA can release free base i^6Ade. The glycosyl bond of i^6Ado is susceptible to acid hydrolysis and cleavage can occur even when a nucleoside is an integral part of the tRNA molecule. In addition to the glycosyl bond cleavage, i^6Ado can undergo a secondary, acid catalyzed reaction in which two products are formed: N^6-(3-hydroxy-3-methylbutyl)adenine and 3H-7,7-dimethyl-7,8,9-trihydropyrimido-[2,1-i]purine (Robbins et al., 1967). The former product has cytokinin activity (Hall and Srivastava, 1968). If the hydrolysis conditions are favourable, which seems to be the case in the acid extraction procedure, the predominent product is the free base i^6Ade [see Martin and Reese, (1968)for comment on this hydrolysis step]. Matsubara et al. (1968), using a mild heating and acidification step, liberated i^6Ade from C. fascians tRNA.

To test whether i^6Ade was liberated from C. fascians tRNA under the conditions of extraction that were used, C. fascians tRNA that had been labelled with [8 -^{14}C] adenine was subjected to heating and acidification procedures simulating those to which cultures of C. fascians

were subjected during the extraction procedure(Fig.33). [8-^{14}C] i^{6}Ade was liberated from the tRNA under these conditions. Therefore, I conclude that there is a real difference in the amount of i^{6}Ade that is extracted from cultures of C. fascians under the two different conditions of extraction. The additional i^{6}Ade that is extracted from the cultures after heating and mild acidification results from the release of i^{6}Ade from the tRNA of these bacteria.

With a clarification of the amount of i^{6}Ade present in the C. fascians cultures this cytokinin is now placed in perspective a propos other cytokinin components. The bioassay results confirm the presence of several cytokinins in the cultures as shown by Klambt et al. (1966). According to my results, however, i^{6}Ade is one of the lesser components accounting for about 20% of the total cytokinin activity. It is very difficult to quantitate the absolute amounts of cytokinin activity present in extracts of biological material by bioassay techniques. It is possible, however, to obtain a rough estimate of the amount of activity present. The activity of the compounds that chromatograph with i^{6}Ade was compatible with the quantity of i^{6}Ade estimated to be present by gas chromatographic techniques.

I have not identified the other cytokinin compounds but the biological activity with respect to column chromatography on both partition and LH-20 column systems coincides with the mobility of i^{6}Ado, ribosyl zeatin and zeatin. None of these compounds was detected on gas chromatography. This can be explained by the fact that the great potency of zeatin means that the absolute amount present in the extracts is less than the amount of i^{6}Ade, and is therefore below the threshold of our

chemical detection procedures. The other cytokinin materials, if they were ribosyl zeatin and i^6Ado, would be very potent cytokinins having almost the molar activity that i^6Ade has. As these compounds represent relatively small proportions of the activity it is likely that they would not be detected on gas chromatography in the small amounts present. The development of a micro bioassay system by Dyson et al. (1970) has enabled us to detect very small quantities of cytokinin activity, and is at present more sensitive than the chemical procedures employed in this work.

The results reported in this thesis suggest that the relationship between free cytokinins and i^6Ado in tRNA is probably indirect. However, tRNA may act as a reservoir of i^6Ado that is subsequently transformed to other cytokinins. Certainly the role of i^6Ade in the pathogenesis of fasciation disease by C. fascians must be a minor one compared with the total cytokinin activity in the cultures, some of which cannot come directly from the tRNA.

(iv) General discussion

In this section the results are discussed in relation to some of the broad problems in the area.

Much work has been done on the metabolism of synthetic cytokinins (McCalla et al., 1962; Guern, 1966; Dyson et al., 1972). It has been suggested that the active compound in plants is a cytokinin metabolite (Dyson, 1969; Dyson et al., 1972). If this is so, then the response of an organism to a cytokinin may depend on its ability to metabolize the cytokinin. A similar situation may exist in animals. Metabolism of

i^6Ado by human and other bone marrow has been studied *in vitro* (Hall and Mintsioulis, in press; Hall *et al.*, 1971). In these tissues an adenosine aminohydrolase activity catalyzes the transformation of i^6Ado to inosine.

In mouse Sarcoma S-180 cells another metabolic pathway exists. Using [8 -^{14}C] i^6Ado, Hakala and her colleagues have shown that the majority of this nucleoside is rapidly converted to the corresponding 5' monophosphate (i^6AMP) (Slocum *et al.*, 1972). Inhibition of adenylate kinase by i^6AMP causes a decrease in ATP levels, leading to cell death.

The primary role of the enzyme activity in 6410 cells that degrades i^6Ado to i^6Ade is one of detoxification. However, degradation of i^6Ado in human and other bone marrow is to inosine (Hall and Mintsioulis, in press; Hall *et al.*, 1971). One possible reason for the existence of these different pathways is that i^6Ado or its metabolites have different roles in different cells. When the relative importance of the various metabolic pathways in a number of tissues is determined this point will be clarified. For example, it may be necessary for cells to contain a small amount of i^6AMP as a physiological regulator. Degradative systems in the cells might then assist in regulating the quantity of this metabolite present in the cells.

N^6-(Δ^2-Isopentenyl)adenosine at concentrations of 3 μM or more inhibits the growth of 6410 cells, and also of tobacco pith tissue (Fig.7,8,20). Whereas I found no evidence of differentiation in 6410 cells that had been inhibited by i^6Ado, the KX-4 tissue did differentiate. Growth of KX-4/R1 tobacco pith tissue was only slightly inhibited by high concentrations of i^6Ado and did not undergo organogenesis.

I have proposed an hypothesis that links the growth responses of KX-4 and KX-4/R1 tissue to i^6Ado to their ability to differentiate. Tissue growth requires energy. However, organogenesis also requires energy, not only for growth of tissue but also to increase the order of the tissue. In normal tobacco tissue, after meristemoid formation, there is considerable growth of new organ tissue. The energy demand on the tissue is then very high and hydrolysis of starch stores is necessary. The growth of KX-4/R1 tissue is not inhibited by i^6Ado. Therefore, more energy must be diverted into the growth processes than normal tissue, and thus there is less energy available for increasing the order of the system. This situation is compounded because the energy available to normal cells from the metabolism of starch is not available to KX-4/R1 tissue, which does not hydrolyse its starch stores. Therefore the reduced energy available to KX-4/R1 tissue prevents it from differentiating.

Gibberellins prevent starch accumulation in tobacco pith tissue that has been treated with cytokinins (Thorpe and Murashige, 1970). Without hydrolysis of starch stores organogenesis does not occur. Therefore, gibberellins antagonize the actions of cytokinins in promoting differentiation. However, growth of tobacco tissue without differentiation requires less energy expenditure and no starch hydrolysis. Thus gibberellins should not adversely affect cytokinin stimulated tissue growth. This is in accordance with the findings of Nitsch (1968).

The mechanism by which i^6Ado inhibits the growth of plant cells is unknown. Hakala and her colleagues (Slocum et al., 1972) have demonstrated in Sarcoma S-180 cells that i^6Ado is converted to i^6AMP,

which inhibits adenylate kinase and reduces the level of ATP. From the "energy-hypothesis" above it is reasoned that cytokinins do not reduce the energy available to plant cells, but increase it to allow differentiation to occur. Therefore, it is unlikely that i^6Ado inhibits plant and mammalian cells by a similar mechanism.

Another question that pertains to both the metabolism of cytokinins and to their mechanism of action is the relationship between free cytokinins and the hypermodified cytokinin nucleosides in the tRNA. The possible inter-relationships were discussed in the Introduction (page 21).

If tRNA were the source of free cytokinins one might expect a feedback control mechanism in which the biosynthesis of i^6Ado and its analogues in the cells was controlled by the quantity of free cytokinins in the cells. No evidence of this was observed in 6410 cells, even when concentrations of i^6Ado only slightly below those toxic to the cells were added to the cultures.

This is analogous to the situation in plant tissues. Plant cells that require cytokinin for growth when grown in the presence of high concentrations of a synthetic cytokinin biosynthesize their own naturally occurring cytokinins in the tRNA (Burrows *et al.*, 1971; Chen and Hall, 1968).

Further evidence that there is not a direct relationship between cytokinins in tRNA and free cytokinins comes from the experiments using *C. fascians*. Matsubara *et al.* (1968) reported the presence of only one cytokinin in the tRNA of *C. fascians*. They identified this cytokinin as i^6Ade by chromatography. However, I found that there were at least four cytokinin activities present in *C. fascians* cultures. One was i^6Ade.

It is possible that the others are metabolically related. In one organism there is direct evidence for this. Miura and Miller (1969) have shown that i^6Ade is converted to zeatin by the fungus Rhizopogon roseolus.

The hypothesis that free cytokinins are not derived directly from the tRNA is supported by other evidence: The cis isomer of zeatin is found in tRNA whereas the trans isomer is found as a soluble cytokinin. The trans isomer is a considerably more active cytokinin than the cis isomer (Skoog and Armstrong, 1970). Although no isomerase activity that interconverts cis and trans zeatin has been isolated, it is possible that such an enzyme exists.

On this evidence, I propose that free cytokinin nucleosides are not derived directly from the hypermodified cytokinin nucleosides of tRNA. However, tRNA may act as a store of relatively inactive cytokinins that are converted to more active cytokinins under appropriate physiological circumstances. This would enable the organism to exert control over the cytokinin activity in its tissues even when there is constant turnover of tRNA.

REFERENCES

REFERENCES

Adams, A., Fitzgerald, P.H. and Gunz, F.W. (1961) A new chromosome abnormality in chronic granulocytic leukaemia. Brit. Med. J., 2, 1474.

Agarwal, M.K. and Weinstein, I.B. (1970) Modifications of ribonucleic acid by chemical carcinogens. II. In vivo reaction of N-2-acetylaminofluorine with rat liver ribonucleic acid. Biochemistry, 9, 503-508.

Armstrong, D.J., Skoog, F., Kirkegaard, L.H., Hampel, A.E., Bock, R.M., Gillam, I. and Tener, G.M. (1969a) Cytokinins: distribution in species of yeast transfer RNA. Proc. Nat. Acad. Sci. U.S., 63, 504-511.

Armstrong, D.J., Burrows, W.J., Skoog, F., Roy, K.L. and Soll, D. (1969b) Cytokinins: distribution in transfer RNA species of Escherichia coli. Proc. Nat. Acad. Sci. U.S., 63, 834-841.

Armstrong, D.J., Evans, P.K., Burrows, W.J., Skoog, F., Petit, J.F., Dahl, J., Steward, T., Strominger, J.L., Leonard, N.J., Hecht, S.M., and Occolowitz, J. (1970) Cytokinins. Activity and identification in Staphylococcus epidermidis transfer RNA. J. Biol. Chem., 245, 2922.

Baikie, A.G., Court Brown, W.M., Jacobs, P.A. and Milne, J.S. (1959) Chromosome studies in human leukaemia. Lancet, 2, 425.

Baikie, A.G., Court Brown, W.M., Buckton, K.E., Harnden, D.G., Jacobs, P.A. and Tough, I.M. (1960) A possible specific chromosome abnormality in human chronic myeloid leukaemia. Nature, 188, 1165.

Barrell, B.G. and Sanger, F. (1969) The sequence of phenylalanine tRNA from E. coli. FEBS Letters, 3, 275-278.

Bartz, J.K., Kline, L.K. and Soll, D. (1970) N^6-(Δ^2-Isopentenyl)adenosine: biosynthesis in vitro in transfer RNA by an enzyme purified from Escherichia coli. Biochem. Biophys. Res. Commun., 40, 1481-1487.

Bessey, O.A., Lowry, O.H. and Brock, M.J. (1946) A method for rapid determination of serum alkaline phosphatase with five cubic millimeters of serum. J. Biol. Chem., 164, 321-329.

Biemann, K., Tsunakawa, S., Sonnenbichler, J., Feldman, H., Dütting, D. and Zachau, H.G. (1966) Struktur eines ungewöhnlichen Nucleosids aus serin-spezifischer Transfer Ribonucleinsäure. Angew. Chem., 78, 600-601.

Burrows, W.J., Skoog, F. and Leonard, N.J. (1971) Isolation and identification of cytokinins located in the transfer ribonucleic acid of tobacco callus grown in the presence of 6-benzylaminopurine. Biochemistry, 10, 2189-2194.

Carr, D.H. and Walker, J.E. (1961) Carbol fuchsin as a stain for human chromosomes. Stain Technology, 36, 233-236.

Chen, C.-M. and Hall, R.H. (1969) Biosynthesis of N^6-(Δ^2-isopentenyl) adenosine in the transfer RNA of cultured tobacco pith tissue. Phytochemistry, 8, 1687.

Chheda, G.B. (1970) Purine and pyrimidine derivatives excreted in human urine. In: Handbook of Biochemistry, 2nd edition, edited by H.A. Sober, Chemical Rubber Company, Cleveland, Ohio, pp. G-106-113.

Divekar, A.Y. and Hakala, M.T. (1971) Adenosine kinase of Sarcoma-180 cells. N^6-substituted adenosines as substrates and inhibitors. Molecular Pharmacology, 7, 663-673.

Dougall, D.K. (1962) On the fate of tyrosine in tobacco callus tissue. Aust. J. Biol. Sci., 15, 619-622.

Dyson, W.H., Chen, C.M., Alam, S.N., Hall, R.H., Hong, C.I. and Chheda, G.B. (1970) Cytokinin activity of ureidopurine derivatives related to a modified nucleoside found in transfer RNA. Science, 170, 328-330.

Dyson, W.H., Fox, J.E. and McChesney, J.D. (1972) Short term metabolism of urea and purine cytokinins. Plant Physiol., 49, 506.

Dyson, W.H. and Hall, R.H. (1972) Cytokinins of autonomous tobacco callus. Submitted to Plant Physiol.

Feder, N. and O'Brien, T.O. (1968) Plant microtechnique: some principles and new methods. Am. J. Bot., 55, 123.

Fittler, F. and Hall, R.H. (1966) Selective modification of yeast seryl-t-RNA and its effect on the acceptance and binding function. Biochem. Biophys. Res. Commun., 25, 441.

Fittler, F., Kline, L.K. and Hall, R.H. (1968a) Biosynthesis of N^6-(Δ^2-isopentenyl)adenosine. The precursor relationship of acetate and mevalonate to the Δ^2-isopentenyl group of transfer ribonucleic acid of microorganisms. Biochemistry, 7, 940-944.

Fittler, F., Kline, L.K. and Hall, R.H. (1968b) N^6-(Δ^2-isopentenyl) adenosine: biosynthesis *in vitro* by an enzyme extract from yeast and rat liver. Biochem. Biophys. Res. Commun., 31, 571.

Fitzgerald, P.H., Adams, A. and Gunz, F.W. (1963) Chronic granulocytic leukaemia and the Philadelphia chromosome. Blood, 21, 183.

Fleysher, M.H., Bloch, A., Hakala, M.T. and Nichol, C.A. (1969) Synthesis and biological activity of some new N^6-substituted purine nucleosides. J. Med. Chem., 12, 1056-1061.

Ford, C.E., Jacobs, P.A. and Lajtha, L.G. (1958) Human somatic chromosomes. Nature, 181, 1565.

Fox, J.E. (1964) Incorporation of kinins into the RNA of plant tissue cultures. Plant Physiol., 37, (Suppl.)

Fox, J.E. (1965) Characterization of labelled RNA from tissues grown on the kinin, N_6-benzyladenine-benzyl-^{14}C. Plant Physiol., 40, IXXVii.

Fox, J.E. (1966) Incorporation of a kinin, N_6-benzyladenine into soluble RNA. Plant Physiol., 41, 75-82.

Fox, J.E. and Chen, C.M. (1967) Characterization of labelled ribonucleic acid from tissue grown on ^{14}C-containing cytokinins. J. Biol. Chem., 242, 4490-4494.

Fox, J.E. and Chen, C.M. (1968) Cytokinin incorporation into RNA and its possible role in plant growth. In: Biochem. and Physiol. of Plant Growth Substances, Editors F. Wightman and G. Setterfield, Runge Press Ltd., Ottawa, p. 777.

Fujita, H. (1939) Uber die Microbestimmung der Blut Phosphatase. J. Biochem. Japan, 30, 69.

Gallo, R.C., Whang-Peng, J. and Perry, S. (1969) Isopentenyl adenosine stimulates and inhibits mitosis of human lymphocytes treated with phytohemagglutinin. Science, 165, 400-402.

Gefter, M. and Russell, R.L. (1969) Role of modifications in tyrosine transfer RNA: a modified base affecting ribosome binding. J. Mol. Biol., 39, 145.

Girard, M. (1967) Isolation of ribonucleic acids from mammalian cells and animal viruses. Methods in Enzymology, 12 (part A), 581-588.

Grace, J.T., Hakala, M.T., Hall, R.H. and Blakeslee, J. (1967) N^6-substituted adenine derivatives as growth inhibitors of human leukaemic myeloblasts and S-180 cells. Proc. Am. Assoc. Cancer Res., 8, 23.

de Gruchy, G.C. (1970) "Clinical Haematology in Medical Practice", 3rd edition, Blackwell Scientific Publications, Oxford and Edinburgh, pp. 360-361.

Guern, J. (1966) Sur les relations entre metabolism et transport de la 6-benzylaminopurine-a^{14}C injectee dans les Jeunes plantes etiolees de Cicer Arientinum L. Comptes rendus, 262, Serie D, p. 2340.

Gunning, B. and Barkley, W. (1963) Kinin-induced directed transport and senescence in detached oat leaves. Nature, 199, 262-265.

Haberlandt, G. (1913) Zur Physiologie der Zellteilung. Sitz. Ber. K. Preuss. Akad. Wiss. 1913, 318-345.

Haberlandt, G. (1921) Wundhormone als Erreger von Zellteilungen. Beite. Allg. Bot., 2, 1-53.

Hall, R.H. (1967) Nucleic acids. Part A. In: Methods in Enzymology, Vol. XII, edited by L. Grossman and K. Moldave, Academic Press, New York, p. 305.

Hall, R.H. (1970) N^6-(Δ^2-Isopentenyl)adenosine: Chemical Reactions, Biosynthesis, Metabolism, and Significance to the Structure and Function of tRNA. In: Progress in Nucleic Acid Research and Molecular Biology, edited by J.N. Davidson and W.E. Cohn, Academic Press, New York, Vol. 10, pp. 57-86.

Hall, R.H. (1971) "The modified nucleosides in nucleic acids", Columbia University Press.

Hall, R.H., Robins, M.J., Stasiuk, L. and Thedford, R. (1966) Isolation of N^6-(γ,γ-Dimethylallyl)adenosine from soluble ribonucleic acid. J. Amer. Chem. Soc., 88, 2614.

Hall, R.H. and Srivastava, B.I.S. (1968) Cytokinin activity of compounds obtained from soluble RNA. Life Sci., 7, 7.

Hall, R.H., Alam, S.N., McLennan, B.D., Terrine, C. and Guern, J. (1971) N^6-(Δ^2-Isopentenyl)adenosine: its conversion to inosine, catalyzed by adenosine aminohydrolases from chicken bone marrow and calf intestinal mucosa. Can. J. Biochem., 49, 623-630.

Hall, R.H. and Mintsioulis, G. (1972) Enzymic activity that catalyzes the degradation of N^6-(Δ^2-isopentenyl)adenosine. Submitted to J. Biochem. Tokyo.

Hampton, A., Biesele, J.J., Moore, A.E. and Brown, G.B. (1956) 6-furfurylamino-9-β-D-ribofuranosylpurine synthesis and differential toxicity to mammalian cells in vitro. J. Am. Chem. Soc. 78, 5695.

Hanoune, J. and Agarwal, M.K. (1970) Studies on the half life time of rat liver tRNA species. FEBS Letters, 11, 78-80.

Hecht, S.M., Leonard, N.J., Burrows, W.J., Armstrong, D.J., Skoog, F. and Occolowitz, J. (1969) Cytokinin from wheat germ transfer RNA: 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine. Science, 166, 1272.

Helgeson, J.P. and Leonard, N.J. (1966) Cytokinins: identification of compounds isolated from Corynebacterium fascians. Proc. Nat. Acad. Sci. U.S., 56, 60-63.

Iwakata, S. and Grace, J.T. (1964) Cultivation in vitro of myeloblasts from human leukaemia. N.Y. J. Med., 64, 2279.

Jablonski, J.R. and Skoog, F. (1954) Cell enlargement of cell division in excised tobacco pith tissue. Physiol. Plant, 7, 16-24.

Jacobs, P.A., Court Brown, W.M., Baikie, A.G. and Strong, J.A. (1959) The somatic chromosomes in mongolism. Lancet, 1, 710.

Jones, R., Grace, J.T., Mittelman, A. and Woodruff, M.W. (1968) Human pharmacology and initial clinical trial of isopentenyl adenosine (IPA). Proc. Am. Assoc. Cancer Res., 9, 35.

Kinlough, M.A. and Robson, H.N. (1961) Study of chromosomes in human leukaemia by a direct method. Brit. Med. J., 2, 1052.

Klambt, D., Thies, G. and Skoog, F. (1966) Isolation of cytokinins from Corynebacterium fascians. Proc. Nat. Acad. Sci. U.S., 56, 52-59.

Kline, L.K., Fittler, F. and Hall, R.H. (1969) N^6-(Δ^2-Isopentenyl) adenosine. Biosynthesis in transfer ribonucleic acid in vitro. Biochemistry, 8, 4361.

La Motte, C.E. (1960) The effects of tyrosine and other amino acids on the formation of buds in tobacco callus. Ph. D. Thesis, University of Wisconsin.

Letham, D.S. (1963) Regulators of cell division in plant tissues. I. Inhibitors and stimulants of cell division in developing fruits: their properties and activity in relation to the cell division period. New Zealand J. Bot., 1, 336-350.

Letham, D.S. (1964) Isolation of a kinin from plum fruitlets and other tissues. In: Regulateurs Naturels de la Croissance Vegetale. (Collogue International Gif-sur-Yvette, 1963) Centre National de la Recherche Scientifique, Paris, p. 109.

Letham, D.S. (1968) A new cytokinin bioassay and the naturally occurring cytokinin complex. In: Biochemistry and Physiology of Plant Growth Substances, edited by F. Wightman and G. Setterfield, Runge Press, Ottawa, p. 19.

Letham, D.S., Shannon, J.S. and McDonald, I.R. (1964) The structure of zeatin, a factor inducing cell division. Proc. Chem. Soc. (London) 1964, 230-231.

Letham, D.S. and Miller C.O. (1965) Identity of kinetin-like factors from Zea Mays. Plant Cell Physiol., 6, 355-359.

Madison, J.T. and Kung, H. (1967) Large oligonucleotides isolated from yeast tyrosine transfer RNA after partial digestion with ribonuclease T_1. Biol. Chem., 242, 1324-1327.

Marinos, N. G. (1967) Multifunctional plastids in the meristematic region of potato tuber buds. J. Ultrastruct. Res., 17, 91-113.

Martin, D.M.G. and Reese, C.B. (1968) Some aspects of the chemistry of N(1)-and N(6)-dimethylallyl derivatives of adenosine and adenine. J. Chem. Soc., (C), 1731.

Matsubara, S., Armstrong, D.J. and Skoog, F. (1968) Cytokinins in tRNA of Corynebacterium fascians. Plant Physiol., 43, 451.

McCalla, D.R., Morre', D.J. and Osborne, D. (1962) The metabolism of a kinin, benzyladenine. Biochem. Biophys. Acta, 55, 522-528.

Miller, C.O. (1961) A kinetin-like compound in maize. Proc. Nat. Acad. Sci. U.S., 47, 170-174.

Miller, C.O. (1962) Additional information on the kinetin-like factor in maize. Plant Physiol., 37, (Suppl.)

Miller, C.O., Skoog, F., Von Saltza, M.H. and Strong, F.M. (1955a) Kinetin, a cell division factor from deoxyribonucleic acid. J. Amer. Chem. Soc., 77, 1329.

Miller, C.O., Skoog, F., Okumura, F.S., Von Saltza, M.H. and Strong, F.M. (1955b) Structure and synthesis of kinetin. J. Amer. Chem. Soc., 77, 2662.

Miller, C.O. and Witham, F.H. (1964) A kinetin-like factor from maize and other sources. In: Regulateurs Naturels de la Croissance Vegetale. (Collogue International Gif-sur-Yvette, 1963) Centre National de la Recherche Scientifique, Paris, p. I (erratum).

Miura, G.A. and Miller, C.O. (1969) 6-(γ,γ-Dimethylallylamino)purine as a precursor of zeatin. Plant Physiol., 44, 372.

Mothes, K. (1960) Uber des altern der blatter und moglichkeit ihrer wiederverjungung. Naturwiss., 47, 337-350.

Murashige, T. (1961) Suppression of shoot formation in cultured tobacco cells by gibberellic acid. Science, 134, 280.

Murashige, T. (1964) Analysis of the inhibition of organ formation in tobacco tissue culture by gibberellin. Physiol. Plant, 17, 636-643.

Nitsch, J.P. (1968) Studies on the mode of action of auxins, cytokinins and gibberellins at the subcellular level. In: Biochemistry and Physiology of Plant Growth Substances, edited by F. Wightman and G. Setterfield, Runge Press, Ottawa.

Nowell, P.C. and Hungerford, D.A. (1960) A minute chromosome in human chronic granulocytic leukaemia. Science, 132, 1497.

Ogawa, Y., Abe, Y. and Fujioka, K. (1957) Effects of kinetin on division of Yoshida Sarcoma cells. Nature, 180, 983-986.

Osborne, D.J. and McCalla, D.R. (1961) Rapid bioassay for kinetin and kinins using senescing leaf tissue. Plant Physiol., 36, 219-221.

Osborne, D.J. (1962) Effects of kinetin on protein and nucleic acid metabolism in Xanthium leaves during senescence. Plant Physiol. 37, 595-602.

Overbeck, J. van, Conkin, M. and Blakeslee, A. (1941) Factors in coconut milk essential for growth and development of Datura embryos. Science, 94, 350-351.

Paces, V., Werstiuck, E. and Hall, R.H. (1971) Conversion of N^6-(Δ^2-isopentenyl)adenosine to adenosine by enzyme activity in tobacco tissue. Plant Physiol., 48, 775-778.

Paleg, L.G. (1960) Physiological effects of gibberellic acid. I. Carbohydrate metabolism and amylase activity of barley endosperm. Plant Physiol., 35, 293.

Paleg, L.G. (1960) Physiological effects of gibberellic acid. II. On starch hydrolysing enzymes of barley endosperm. Plant Physiol., 35, 902.

Paleg, L.G. (1961) Physiological effects of gibberellic acid. III. Observations on its mode of action on barley endosperm. Plant Physiol., 36, 829.

Peterkofsky, A. (1968) The incorporation of mevalonic acid into N^6-(Δ^2-isopentenyl)adenosine of transfer RNA in Lactobacillus acidophilus. Biochemistry, 7, 472.

Richmond, A. and Lang, A. (1957) Effect of kinetin on protein content and survival of detached Xanthium leaves. Science, 125, 650-651.

Robins, M.J., Hall, R.H. and Thedford, R. (1967) N^6-(^{2}-Isopentenyl) adenosine. A component of the transfer ribonucleic acid of yeast and of mammalian tissue, methods of isolation, and characterization. Biochemistry, 6, 1837.

Rogozinska, J.H., Helgeson, J.P. and Skoog, F. (1964) Tests for kinetin-like growth promoting activities of triacanthine and its isomer, 6(γ,γ-dimethylallylamino)-purine. Physiol. Plant, 17, 165-176.

Sachs, T. and Thimann, K.V. (1964) Release of lateral buds from apical dominance. Nature, 201, 939-940.

Samuels, R.M. (1961) Bacterial-induced fasciation in Pisum sativum var. Alaska. Ph. D. Thesis, Indiana University.

Scherrer, K. and Darnell, J.E. (1962) Sedimentation characteristics of rapidly labelled RNA from HeLa cells. Biochem. Biophys. Res. Commun., 1, 486.

Skoog, F. and Armstrong, D.J. (1970) Cytokinins. In: Annual Review of Plant Physiology. 21, 359-384.

Skoog, F. and Tsui, C. (1948) Chemical control of growth and bud formation in tobacco stem segments and callus cultured in vitro. Amer. J. Bot., 35, 782-787.

Skoog, F. and Miller, C.O. (1957) Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symp. Soc. Exp. Biol. 11, 118-131.

Skoog, F., Hamzi, H.Q., Szweykowska, A.M., Leonard, N.J., Carraway, K.L., Fujii, T., Helgeson, J.P. and Leoppky, R.N. (1967) Cytokinins: structure/activity relationships. Phytochemistry, 6, 1169.

Slocum, H.K., Divekar, A.Y. and Hakala, M.T. (1972) N^6-(Δ^2-Isopentenyl) adenosine (IPAdo); uptake, metabolism, and site of action in Sarcoma-180 cells (S-180). Proc. Am. Assoc. Cancer Res., 13, 70.

Srivastava, B.I.S. (1966) Metabolism of kinetin in excised barley leaves and in tobacco pith tissue culture. Plant Physiol., 41, IXii-IXiii.

Staehelin, M., Rogg, H., Baguley, B.C., Ginsberg, T. and Wehrli, W. (1968) Structure of a mammalian serine tRNA. Nature, 219, 1363.

Steward, F.C., Kent, A.E. and Mears, K. (1958) Growth and organized development of cultured cells. II. Organization in cultures grown from freely suspended cells. Amer. J. Bot., 45, 705-708.

Suk, D., Simpson, C.L. and Mihich, E. (1970) Toxicological and antiproliferative effects of N^6-(Δ^2-isopentenyl)adenosine, a natural component of mammalian tRNA. Cancer Research, 30, 1429-1436.

Syono, K. (1965) Physiological and biochemical changes of carrot root callus during successive cultures. Plant Cell Physiol., 6, 371-392.

Thimann, K.V. and Sachs, T. (1966) The role of cytokinins in the "fasciation" disease caused by Corynebacterium fascians. Amer. J. Bot., 53, 731-739.

Thorpe, T.A. and Murashige, T. (1968) Starch accumulation in shoot-forming callus cultures. Science, 160, 421-422.

Thorpe, T.A. and Murashige, T. (1970) Some histochemical changes underlying shoot initiation in tobacco callus cultures. Can. J. Bot., 48, 277-285.

Torrey, J.G. (1966) The initiation of organized development in plants. Advan. Morphogenesis, 5, 39-91.

Uziel, M. and Gassen, H.G. (1969) Phenylalanine transfer RNA from E. coli B. Isolation and characterization of oligonucleotides from ribonuclease T_1 and ribonuclease A hydrolysates. Biochemistry, 8, 1643-1655.

Valentine, W.N., Follette, J.H., Solomon, D.H. and Reynolds, J. (1957) Biochemical and enzymatic characteristics of normal and leukemic leukocytes with particular reference to leukocyte alkaline phosphatase. In: The Leukemias, Academic Press, New York, p. 457.

Wachstein, M. (1946) Alkaline phosphatase activity in normal and abnormal human blood and bone marrow cells. J. Lab. & Clin. Med., 31, 1.

Wecker, E. (1959) The extraction of infectious virus nucleic acid with hot phenol. Virology, 7, 241.

White, P. (1939) Potentially unlimited growth of excised plant callus in an artificial nutrient. Amer. J. Bot., 26, 59-64.

Wickson, M. and Thimann, K.V. (1958) The antagonism of auxin and kinetin in apical dominance. Physiol. Plant, 11, 62-74.

Wiesner, J. (1892) "Die elementarstruktur und das wachstum der lebenden substanz", A. Holder, Vienna.

Wollgiehn, R. (1965) Kinetin and nucleinsaurestoffwechsel. Flora, 156, 291-302.

Xefteris, E., Mitus, W.J., Mednicoff, I.B. and Dameshek, W. (1961) Leukocytic alkaline phosphatase in busulfan-induced remissions of chronic granulocytic leukaemia. Blood, 18, 202.

Zachau, H.G., Dütting, D. and Feldmann, H. (1966) The structure of two serine transfer ribonucleic acids. Z. Physiol. Chem., 347, 212.

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Metabolism and Biological Effects of N^6-(Δ^2-Isopentenyl)-adenosine on a Cell Line Derived from Circulating Leukocytes of a Patient with Chronic Myelogenous Leukemia[1]

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SUMMARY

N^6-(Δ^2-Isopentenyl)adenosine (i^6Ado) at a concentration of 3 μM inhibits the growth of a line of cells derived from the circulating leukocytes of a patient with myelogenous leukemia, Roswell Park Memorial Institute line 6410. No alteration in the alkaline phosphatase activity of these cells is observed under these conditions.

i^6Ado is a component of 6410 cell tRNA but could not be detected as a nucleoside free in the cells or culture medium. The tRNA of cells cultured in the presence of i^6Ado contains a normal complement of Δ^2-isopentenyl groups.

Line 6410 cells contain an enzyme activity that converts i^6Ado to the corresponding base, which is much less toxic to these cells.

INTRODUCTION

i^6Ado[3] occurs in all species as a component of the tRNA (7). This nucleoside has attracted considerable attention because, in the free form, it exhibits potent physiological properties. In plant systems, for example, it stimulates growth and development [see review by Skoog and Armstrong (17)]. In mammalian systems, the effects seem to be more negative; it inhibits growth of certain lines of cancer cells *in vitro* and *in vivo* (5, 18, 19) as well as of human blood lymphocytes (3).

In the intact organism there is presumably some turnover or breakdown of tRNA, and this would release i^6Ado. The tRNA of rat liver, for example, has a half-life of 5 days (9), and in humans the occurrence of modified components of tRNA in urine is evidence for tRNA turnover (1). In view of its physiological activity, it is perhaps not surprising to find strong enzymic activity that catalyzes the destruction of i^6Ado in human and other animal bone marrow (Ref. 8; R. H. Hall and G. Mintsioulis. Enzymic Activity that Catalyzes Degradition of N^6-(Δ^2-Isopentenyl)adenosine, submitted for publication to *Canadian Journal of Biochemistry*).

Because intact animals have a mechanism for metabolizing any released i^6Ado, we investigated in more detail the inhibition of cultured cells by i^6Ado.

MATERIALS AND METHODS

General. i^6Ado was prepared according to the method of Grimm *et al.* (6). i^6Ado-8-^{14}C (5 mCi/mmole) was synthesized according to the method of Paces *et al.* (13) by Dr. Dennis Whitty, in this laboratory. Adenine-8-^{14}C (52.6 mCi/mmole) was obtained from Schwarz BioResearch, Orangeburg, N. J. Bacterial alkaline phosphatase was obtained from Worthington Biochemical Corp., Freehold, N. J. Mevalonic acid-2-^{14}C (2.95 mCi/mmole) was obtained from New England Nuclear, Boston, Mass.

Cultured Cells. Cell line 6410, derived from a patient with myelogenous leukemia (10), was obtained from Roswell Park Memorial Institute. This cell line, like other lines established from the peripheral blood of patients with chronic myeloid leukemia, is almost certainly not the characteristic cell of the disease. Although the cell line was originally described as myeloblastic (10) it is better described as derived from immature hemopoietic cells. The cells were maintained in culture medium RPMI 1629, containing 15% fetal calf serum. Culture medium and calf serum were obtained from Grand Island Biological Co., Grand Island, N. Y. We determined total cell count and cell viability by staining aliquots of the cultures with trypan blue and counting stained and unstained cells on a hemacytometer (14).

Paper Chromatography. Whatman No. 1 paper was used. The solvent systems were: (*a*) 1-butanol:concentrated ammonium hydroxide:water, 86:5:14; (*b*) 1 M ammonium borate, pH 9.0:95% ethanol, 9:1; (*c*) ethyl acetate:water, 4:1.

Column Chromatography. Partition columns were prepared according to the method of Hall (7), with the use of Solvent C. DEAE-cellulose was obtained from Whatman Co., H. Reeve-Angel, Clifton, N. J.

Alkaline Phosphatase Assays. These were performed by means of the colorimetric method described in Ref. 16 with the use of *p*-nitrophenyl phosphate as a substrate.

Preparation of RNA. RNA was extracted by the method of Girard (4). tRNA was prepared by chromatography of this material on a DEAE-cellulose column eluted with a gradient of sodium chloride, 0.1 to 1.0 M in 0.1 M Tris-Cl, pH 7.2. The

[1] This work was supported by a grant from the National Cancer Institute of Canada and by Grant MT-2738 from the Medical Research Council of Canada.

[2] Research Fellow of the National Cancer Institute of Canada.

[3] The abbreviations used are: i^6Ado, N^6-(Δ^2-isopentenyl)adenosine; i^6Ade, N^6-(Δ^2-isopentenyl)adenine.

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tRNA eluted at a sodium chloride concentration of between 0.4 and 0.7 M.

Preparation of Nucleosides from RNA. The RNA sample was hydrolyzed with 0.3 N KOH for 18 hr at 37°. The pH of the sample was adjusted to 9.2 by the addition of 1 N HCl. One mg of chicken intestinal alkaline phosphatase (Type IV, Sigma) was added per 100 A_{260} units of RNA, and the solution was incubated for 24 hr at 37°.

RESULTS

The Effect of i⁶Ado on the Growth and Viability of 6410 Cells in Culture. The addition of i⁶Ado to cultures of 6410 cells at concentrations below 1.5 μM had no effect on the duration of the lag phase or on the growth rate or viability of the cell cultures. At concentrations of 3 μM or more, this nucleoside arrested the growth of the cells (Chart 1) and caused a progressive decrease in the viability of the cultures.

The Effect of i⁶Ado on Alkaline Phosphatase Activity of 6410 Cells *in Vitro*. Neutrophil alkaline phosphatase is usually reduced or absent in chronic myeloid leukemia but returns to normal during remissions (2). It was therefore relevant to ascertain whether i⁶Ado treatment raised the level of alkaline phosphatase in 6410 cells. Four 200-ml cultures of 6410 cells were grown until early logarithmic phase. One ml of i⁶Ado solution was added to 3 of the cultures so that the final concentrations of i⁶Ado were 0.15, 0.6, and 3 μM respectively One ml of distilled water was added to the control culture. Immediately after the addition of i⁶Ado to the cultures and at subsequent times up to 46 hr, 10-ml samples were removed from each culture. The total cell count and viability of each sample were determined. The cells were harvested by centrifugation at 20,000 X *g* for 20 min at 4°. The cells were resuspended in 2 ml of distilled water and disrupted by sonification, and the homogenates were assayed for alkaline phosphatase activity. No change was observed in the alkaline phosphatase activity as a result of treatment with i⁶Ado.

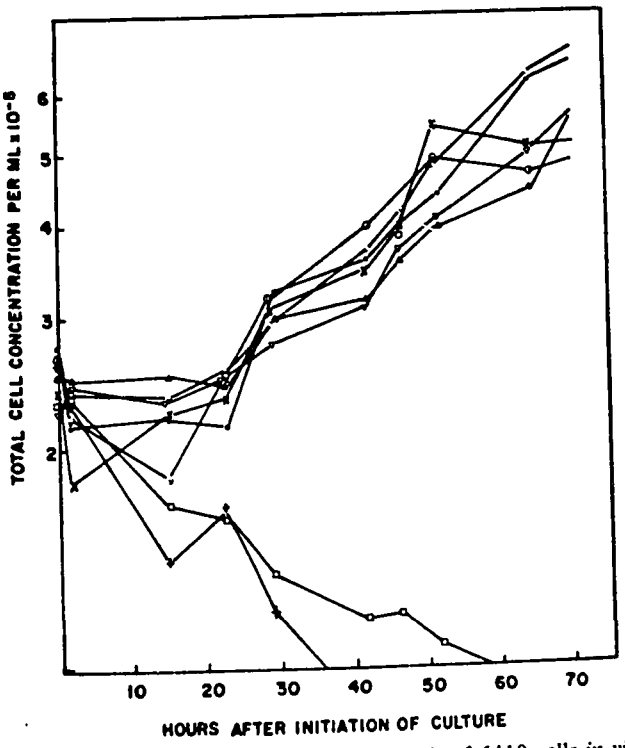

Chart 1. The effect of i⁶Ado on the growth of 6410 cells *in vitro*. Cells were harvested by centrifugation from cultures in logarithmic growth phase and resuspended in fresh medium. Aliquots (10 ml) of this suspension were dispensed into culture bottles containing growth medium (190 ml) in which were dissolved various concentrations of i⁶Ado. The final cell concentration in each was about 2.5 X 10^5 cells/ml, and the viability was 85 to 90%. The total cell concentration of each culture is plotted against time. ⊙, control; ○, control; ×, i⁶Ado, 0.15 μM; ▲, i⁶Ado, 0.3 μM; ▽, i⁶Ado, 0.6 μM; ●, i⁶Ado, 1.5 μM; □, i⁶Ado, 3.0 μM; +, i⁶Ado, 6.0 μM. The numbers along the ordinate should be multiplied by 10^{-5} to obtain the correct values.

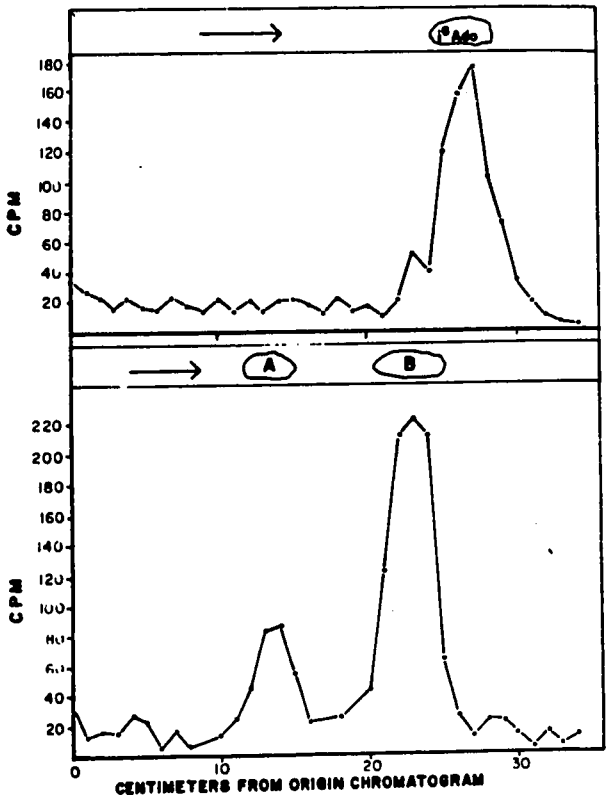

Chart 2. The presence of i⁶Ado in 6410 cell RNA. Three liters of cells in logarithmic phase were labeled with 50 μCi of adenosine-8-^{14}C (specific activity, 52.6 mCi/mmole). After 72 hr, the cells were harvested by centrifugation for 10 min at 10,000 X *g* at 4°. Their RNA was extracted by the method of Girard (4). Higher-molecular-weight RNA was separated from soluble RNA species by precipitation in 1 M sodium chloride and removed by centrifugation. The soluble RNA was precipitated with 2 volumes of cold 95% ethanol, hydrolyzed to its constituent nucleosides, and chromatographed on a partition column in solvent System C (16). The first 35-ml fraction in which i⁶Ado eluted under these conditions was mixed with a sample of i⁶Ado (0.25 mg). *Top*, radioactivity profile of an aliquot of the eluate after paper chromatography in System A; *bottom*, radioactivity profile of an aliquot of the eluate after hydrolysis in 1 N HCl for 15 min at 100°, chromatographed on paper in System A; *i⁶Ado*, position of i⁶Ado marker; *A* and *B*, position of hydrolysis products of i⁶Ado: 3*H*-7,7-dimethyl-7,8,9-trihydropyrimido-[2,1-i] purine, and *N*⁶′-(3-hydroxy-3-methylbutyl)adenine.

The Presence of i⁶Ado in tRNA of 6410 Cells. i⁶Ado or a

derivative has been found as a component of tRNA of all species investigated (7). In plants and some microorganisms this nucleoside or a hydroxylated derivative occurs free either intra- or extracellularly. Because i⁶Ado is toxic to 6410 cells, studies were done to determine whether i⁶Ado is a constituent of the cells. The results of experiments described in Chart 2 show that 6410 cells contain i⁶Ado in the tRNA. However, no i⁶Ado was detected in the RNA fraction which was insoluble in 1 M sodium chloride.

Assuming that all the label was incorporated into the nucleic acids as adenine derivatives and that these constitute approximately 25% of the bases in 6410 cell RNA, we calculate that i⁶ Ado constitutes about 0.034% of the total bases in the soluble RNA, a value that agrees with the level found in RNA from other sources (15).

i⁶Ado in the Free State. The possibility that 6410 cells contained i⁶Ado free in their cytoplasm or that they released the nucleoside into the medium was investigated.

Four liters of 6410 cells, growing logarithmically, were labeled with 50 μCi of adenosine-8-^{14}C (specific activity, 52.6 mCi/mmole). After 48 hr, the cells were harvested by centrifugation for 10 min at 3000 × *g* at 0°, resuspended in 10 ml of 10 mM Tris-Cl buffer (pH 7.5), and disrupted by ultrasound. Unlabeled i⁶Ado (0.25 mg) was added to the homogenate. The macromolecules were precipitated by the addition of 10 ml of cold 10% perchloric acid and were removed by centrifugation. The supernatant was neutralized and chromatographed on a partition column in solvent C (15). The first 35-ml fraction, in which both i⁶Ado and i⁶Ade elute, was rechromatographed on paper developed in System A. The chromatogram was cut into strips, and the radioactivity was determined by liquid scintillation counting. No evidence for the presence of i⁶Ado or its corresponding base was found.

The culture medium from which the cells had been removed was extracted 4 times with an equal volume of ethyl acetate. This extract was then chromatographed in the same way as the cell extract. No i⁶Ado or i⁶Ade was found.

Table 1

Δ²-Isopentenyl pyrophosphate:tRNA-Δ²-isopentenyltransferase activity in 6410 cells

Three g of 6410 cells were homogenized in a French press in 15 ml of a buffer, 50 mM Tris-Cl, pH 7.5:20 mM mercaptoethanol:5 mM magnesium chloride. The homogenate was centrifuged at 40,000 × *g* for 30 min at 4°. Four-ml aliquots of the supernatant were added to a solution of ATP (50 mg):mevalonic acid-2-^{14}C, 2.15 × 10⁶ cpm, and 100 A_{260} units of tRNA, at pH 7.5, and were incubated for 1 hr at 37°. tRNA was isolated from the reaction mixture by the method of Kline *et al.* (12), and the radioactivity of an aliquot was determined by liquid scintillation counting (efficiency about 70%). The *E. coli* tRNA was hydrolyzed to its constituent nucleosides. All the radioactivity was cochromatographed with i⁶Ado on partition chromatography in System C and on paper in System A. Acid hydrolysis yielded the 2 characteristic products of i⁶Ado.

Enzyme source	tRNA substrate source	cpm/25 A_{260} units of tRNA
6410 cells	*E. coli*	1115.4
6410 cells	6410 cells	52.4
6410 cells	6410 cells grown in the presence of 2.25 μM i⁶Ado for 72 hr	0.0

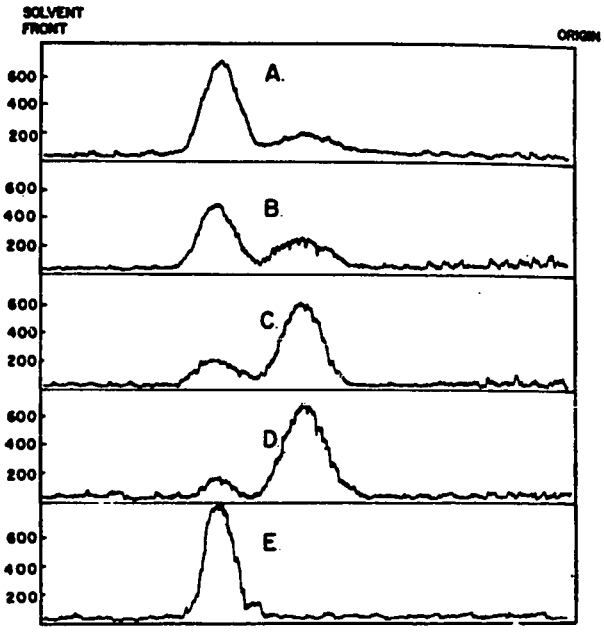

Chart 3. Metabolism of i⁶ Ado-8-^{14}C by an enzyme from 6410 cells. One g of 6410 cells was resuspended in twice its volume of a buffer: 50 mM Tris-Cl:5 mM $MgCl_2$:20 mM 2-mercaptoethanol, pH 7.5. The cells were disrupted sonically, and the homogenate was centrifuged at 20,000 × *g* for 30 min at 4°; 25 μl of the supernatant were added to each tube, which contained 10,000 dpm of i⁶Ado-8-^{14}C (specific activity, 5 mCi/mmole), in 2 ml of a mixture containing 50 mM NaH_2PO_4:0.4 mM $MgCl_2$, pH 6.5. After various intervals, aliquots of the reaction mixture were chromatographed on paper in Solvent System B. The distribution of radioactivity was determined with a radiochromatogram scanner. *A*, 1-hr incubation; *B*, 3-hr incubation; *C*, 16-hr incubation; *D*, 24-hr incubation; *E*, 24-hr incubation (boiled enzyme control).

Δ²-Isopentenyl Pyrophosphate:tRNA-Δ²-Isopentenyltransferase. Kline *et al.* (12) have shown that yeast and mammalian cells contain an enzyme that catalyzes the transfer of Δ²-isopentenyl groups from Δ²-isopentenyl pyrophosphate to preformed tRNA. Because 6410 cells contain i⁶Ado in their tRNA, experiments were done to determine whether this enzymic activity is present.

The results of these experiments (Table 1) indicate that 6410 cells contain an enzyme activity that transfers Δ²-isopentenyl groups to preformed *Escherichia coli* tRNA to form i⁶Ado. This parallels the results obtained with yeast and rat liver (12). Neither tRNA from 6410 cells nor that from 6410 cells grown in the presence of i⁶Ado serves as a substrate for the enzyme.

The Degradation of i⁶Ado by an Enzyme Activity in 6410 Cells. i⁶Ado is a component of tRNA of 6410 cells. If tRNA is degraded, the cells could be exposed to this compound. Experiments were therefore done to determine whether 6410 cells have a mechanism for detoxifying i⁶Ado.

Chart 3 shows that i⁶Ado-8-^{14}C was progressively degraded to a single product. This product cochromatographs with i⁶Ade in Systems A and B, but not with i⁶Ado or its theoretical metabolic products, adenine, adenosine, inosine, hypoxanthine, N^6-(4-hydroxy-3-methylbut-2-enyl)adenosine.

N^6-(3-hydroxy-3-methylbutyl)adenosine, or N^6-(3-hydroxy-3-methylbutyl)adenine.

The identity of the product was confirmed as i^6Ade when on acid hydrolysis it yielded the 2 characteristic breakdown products (15).

DISCUSSION

The growth of cultures of 6410 cells is inhibited by i^6Ado at a concentration of 3 μM. The cells grow normally when the concentration of i^6Ado is 2.0 μM. This indicates that there is a distinct threshold at which this nucleoside exerts toxic effects. Inhibition of growth is associated with an immediate loss of viability. No alteration in the length of the lag phase of the culture was noted when sublethal concentrations of i^6Ado were used.

i^6Ado caused transient hematological and clinical remission in a patient with acute promyelocytic leukemia (11). Gallo *et al.* (3) found that the nucleoside at certain concentrations inhibited the growth of human blood lymphocytes in culture, while at lower concentrations there appeared to be a stimulation of DNA synthesis and mitosis. i^6Ado has no observable stimulatory effects on the growth of 6410 cells *in vitro*. We questioned, however, whether the level of alkaline phosphatase might be an indicator of such effects. Leukocyte alkaline phosphatase is usually reduced or absent in chronic myeloid leukemia, although it returns to normal during remissions (5). No change in the alkaline phosphatase activity of 6410 cells was observed when the cells were cultured in the presence of i^6Ado.

The tRNA of 6410 cells contains a normal complement of i^6Ado, and the biosynthetic pathway for the formation of this nucleoside in tRNA appears to be functioning normally. Exogenously added i^6Ado, at a level slightly below that lethal to 6410 cells, has no apparent effect on this enzyme activity.

i^6Ado, or a closely related derivative, occurs in the free state in some microorganisms and in certain parts of plants [see review by Hall (7)]. The genesis of the free nucleoside is not known, but turnover of the tRNA could represent one source. We asked whether these cells contained the nucleoside or its base in an unbound state. No trace of either form could be detected in the cells or in the culture medium. The limits of detection of free i^6Ado or i^6Ade in the cells or in their medium may be judged from similar experiments with *Corynebacterium fascians*, (M. P. Rathbone and R. H. Hall, manuscript in preparation). i^6Ade was detected in cultures of this organism at a concentration of 1.5 nM by means of a similar analytical procedure.

6410 cells contain an enzyme activity that catalyzes deletion of the sugar residue to give i^6Ade. The enzymatic activity that catalyzes conversion of i^6Ado to inosine in bone marrow (R. H. Hall and G. Mintsioulis. Enzymic Activity that Catalyzes Degradation of N^6-(Δ^2-Isopentenyl)adenosine, submitted for publication to *Canadian Journal of Biochemistry*. See also Ref. 8), and to adenosine in plants (15) was not detected. Since the base is at least 50 times less inhibitory to 6410 cells than the parent nucleoside (3), the enzyme activity represents a mechanism by which the cells nullify the inhibitory effects of i^6Ado.

ACKNOWLEDGMENTS

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REFERENCES

1. Chheda, G. B. Purine and Pyrimidine Derivatives Excreted i Human Urine. *In:* H. A. Sober (ed.), Handbook of Biochemistr Ed. 2, pp. G-106–G-113. Cleveland: Chemical Rubber Co., 1970.
2. de Gruchy, G. C. Clinical Haematology in Medical Practice, Ed. pp. 360–361. Oxford, England: Blackwell Scientific Publication 1970.
3. Gallo, R. C., Whang-Peng, J., and Perry, S. Isopentenyl-adenosin Stimulates and Inhibits Mitosis of Human Lymphocytes Treate with Phytohemagglutinin. Science, *165:* 400–402, 1969.
4. Girard, M. Isolation of Ribonucleic Acids from Mammalian Cel and Animal Viruses. Methods Enzymol., *12* (Part A): 581–58 1967.
5. Grace, J. T., Hakala, M. T., Hall, R. H., and Blakeslee, J N^6-Substituted Adenine Derivatives as Growth Inhibitors c Human Leukemic Myeloblasts and S-180 Cells. Proc. Am. Asso Cancer Res., *8:* 23, 1967.
6. Grimm, W., Fujii, T., and Leonard, N. J. Preparation c N^6-Substituted Adenosines by Alkylation at the 1-Position o Adenosine followed by Rearrangement. *In:* W. W. Zorbach and R S. Tipson (eds.), Synthetic Procedures in Nucleic Acid Chemistry pp. 212–214. New York: Interscience Publishers, Inc., 1968.
7. Hall, R. H. The Modified Nucleosides in Nucleic Acids. New York Columbia University Press, 1971.
8. Hall, R. H., Alam, S. N., McLennan, B. D., Terrine, C., and Guern J. N^6-(Δ^2-Isopentenyl)adenosine: Its Conversion to Inosin Catalyzed by Adenosine Aminohydrolases from Chicken Bon Marrow and Calf Intestinal Mucosa. Can. J. Biochem., *49* 623–630, 1971.
9. Hanoune, J., and Agarwal, M. K. Studies on the Half Life Time o Rat Liver tRNA Species. Federation European Biochem. Soc Letters, *11:* 78–80, 1970.
10. Iwakata, S., and Grace, J. T., Jr. Cultivation *in Vitro* o Myeloblasts from Human Leukemia. N. Y. State J. Med., *64* 2279–2282, 1964.
11. Jones, R., Grace, J. T., Mittelman, A., and Woodruff, M. W. Huma Pharmacology and Initial Clinical Trial of Isopentenyl Adenosin (IPA). Proc. Am. Assoc. Cancer Res., *9:* 35, 1968.
12. Kline, L. K., Fittler, F., and Hall, R. H. N^6-(Δ^2-Isopentenyl)adeno sine. Biosynthesis in Transfer Ribonucleic Acid *in Vitro* Biochemistry, *8:* 4361–4371, 1969.
13. Paces, V., Werstiuk, E., and Hall, R. H. Conversion o N^6-(Δ^2-Isopentenyl)adenosine to Adenosine by Enzyme Activity in Tobacco Tissue. Plant Physiol., *48:* 775–778, 1971.
14. Paul, J. Cell and Tissue Culture, Ed. 4, pp. 356–357. Edinburgh E. & S. Livingstone, 1970.
15. Robins, M. J., Hall, R. H., and Thedford, R. N^6-(Δ^2-Isopentenyl) adenosine. A Component of the tRNA of Yeast and of Mammalia Tissue, Methods of Isolation, and Characterization. Biochemistry *6:* 1837–1848, 1967.
16. Sigma Technical Bulletin No. 104. St. Louis, Mo.: Sigma Chemic Co., March 1963.
17. Skoog, F., and Armstrong, D. J. Cytokinins. Ann. Rev. Plan Physiol., *21:* 359–384, 1970.
18. Slocum, H. K., Divekar, A. Y., and Hakala, M. T. N^6-(Δ^2-Is pentenyl)adenosine (IPAdo): Uptake, Metabolism and Site o Action in Sarcoma 180 Cells (S-180). Proc. Am. Assoc. Cance Res., *13:* 70, 1972.
19. Suk, D., Simpson, C. L., and Mihich, E. Toxicological an Antiproliferative Effects of N^6-(Δ^2-Isopentenyl)adenosine, Natural Component of Mammalian tRNA. Cancer Res., 30 1429–1436, 1970.

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Concerning The Presence of The Cytokinin, N^6-(Δ^2-Isopentenyl)adenine, In Cultures of *Corynebacterium fascians*

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SUMMARY

Corynebacterium fascians causes a fasciation disease in a number of dicotyledons and this disease appears to be caused by compounds with cytokinin activity elaborated by the infecting bacteria. Extractions of *C. fascians* in late logarithmic phase under conditions where the pH never falls below 7.0 yield about 2 µg/l of N^6-(Δ^2-isopentenyl)adenine, a potent cytokinin. If a mild acidification step is included in the extraction procedure the yield increases to about 12 µg/l. This is due to release of N^6-(Δ^2-isopentenyl)adenine from *C. fascians* tRNA during the extraction procedure. In terms of total cytokinin activity present in *C. fascians* cultures, N^6-(Δ^2-isopentenyl)adenine appears to be a minor component.

FOOTNOTE

1. Abbreviations

i^6Ade	=	$\underline{N}^6$-(Δ^2-isopentenyl)adenine
i^6Ado	=	$\underline{N}^6$-(Δ^2-isopentenyl)adenosine
BSTFA	=	$\underline{N}$,$\underline{O}$-Bis(trimethylsilyl)trifluoroacetamide
GLC	=	Gas-liquid chromatography

INTRODUCTION

Corynebacterium fascians is a plant pathogen that causes a "fasciation" disease in a number of dicotyledonous seedlings (Roussaux, 1965; Thimann and Sachs, 1966). The symptoms of this disease are mimicked by injection of cytokinins into the stems of plants, and this observation led to the suggestion that the disease is caused by cytokinins elaborated by the infecting bacteria. Thimann and Sachs (1966) found such cytokinin activity in cultures of *C. fascians*. Klämbt *et al.* (1966) extracted N^6-(Δ^2-isopentenyl)adenine[1] from cultures of *C. fascians* and reported the presence of other cytokinins of undetermined identity. Matsubura *et al.* (1968) showed that the tRNA of *C. fascians* contained component(s) with cytokinin activity and on this basis suggested that N^6-(Δ^2-isopentenyl)adenosine[1] is a component of the tRNA.

Knowledge of the biogenesis of free i^6Ade would contribute towards an understanding of the role of cytokinins in growth and development. N^6-(Δ^2-isopentenyl)adenine is synthesized in preformed tRNA by addition of the Δ^2-isopentenyl group (see review by Hall (1971). The free cytokinin could be released on breakdown of tRNA or it could be synthesized by a totally independent pathway. We originally set out to investiga these possibilities using *C. fascians* as a model, but before we could proceed it was necessary to confirm the presence of free i^6Ade in the culture and at what level. Also it was necessary to clarify some general points about technique.

Materials and Methods

C. fascians cultures were a gift from Professor J. Guern, Physiologie Végétale, Sorbonne, Paris. The pathogenicity of this strain was confirmed by its ability to induce fasciation in pea seedlings. The culture medium had the same formulation as that used by Klämbt et al. (1966). C. fascians were cultured in 2 litre batches in erlenmeyer flasks of 4 litre capacity which were aerated by shaking in a water bath (New Brunswick Scientific) maintained at 27°. The cultures were grown to stationary phase before harvesting. In experiments in which extraction techniques were compared, the cultures were first pooled, mixed, and divided into two equal parts.

Bioassay for cytokinin activity was carried out using a soybean tissue culture assay (Dyson et al, 1970).

Sephadex LH-20 was obtained from Pharmacia. Fifty gm of LH-20 was prepared according to the manufacturer's instructions using 35% aqueous ethanol as a solvent and poured into a column 2.5 cm x 50 cm.

Partition columns were prepared using Celite 545 (Johns Manville brand of diatomaceous earth) according to the method of Hall (1971). The columns had a diameter of 1.9 cm and contained 40 g of Celite 545 mixed with 20 ml of the lower phase of solvent A. The sample to be chromatographed was dissolved in 5 ml of lower phase of solvent A and mixed with 10 g of Celite 545 and packed onto the top of the column which was then developed with upper phase of solvent A.

Paper chromatography was carried out at room temperature in sealed glass tanks using descending chromatography on whatman No. 1 paper.

The solvent systems were: A, ethyl acetate:water, 4:1, B, 1-butanol:concentrated ammonium hydroxide:water, 86:5:14.

GLC separation was carried out on an F & M Scientific dual-column gas chromatograph, model 402, equipped with flame-ionization detectors. A 0.3 x 120 cm U-shaped silanized glass column packed with 10% DC-11 (w/w) on Gas Chrom Q was conditioned for 48 hr before use. Carrier gas (nitrogen) flow was 35 ml/min.

Total RNA was extracted from C. fascians by the method of Girard (1967). tRNA was purified from this by chromatography on DEAE cellulose columns using a linear salt gradient from 0.1 to 1.0 M.

N^6-(Δ^2-isopentenyl)adenine was obtained from Raylo Chemicals, Canada. BSTFA was obtained from Regis Chemical Co., Chicago, Illinois. Dow-Corning silicone grease DC-11 and Gas Chrom Q were purchased from Kenscott Ltd., Toronto, Ontario, and Applied Science Laboratories, State College, Pennsylvania, respectively.

[8-^{14}C]Adenine, specific activity of 50.5 µCi/µmole, was obtained from Amersham Searle Corporation.

Radioactivity of paper chromatograms was determined by cutting the chromatogram into one cm strips. Each strip was placed in a 15 ml solution of toluene that contained 5 g per litre of 2,5-diphenyloxazole and 0.1 g per litre of p-bis 2-(5-thenyloxazolyl)-benzene using a Nuclear Chicago Mark II liquid scintillation counter.

RESULTS

Extraction of Cytokinins from Cultures of C. fascians. Two variations in the initial part of the procedure were used. A four litre culture of C. fascians grown to stationary phase was divided into equal parts.

Procedure 1. The culture was heated for 90 min at 95° in a water bath, and then acidified with 16 ml of concentrated hydrochloric acid per li The culture was allowed to cool for 30 min so that the bacteria precipitated leaving a clear supernatant. The supernatant was decanted and the precipitate washed with 35% aqueous ethanol, filtered and discarded. The washings and supernatant were pooled, filtered through Celite, neutralized with 1N NH_4OH and reduced to 250 ml on a rotary evaporator in vacuo. The extract was then treated in exactly the same manner as the cultures were treated in procedure 2.

Procedure 2. The culture was extracted by shaking six times with equal volumes of 1-butanol in a separatory funnel. Removal of bacteria by centrifugation before this procedure did not noticeably change the recovery of i^6Ade, and therefore in most of the experiments the bacteria were removed from the culture prior to extraction with 1-butanol. The 1-butanol fractions were pooled and reduced to dryness on a rotary evaporator in vacuo. The resulting material was redissolved in 5 ml of lower phase of solvent A and chromatographed on a 50 g Celite column developed with upper phase of solvent A.

The first 125 ml (this fraction will contain, if present, i^6Ade, i^6Ado zeatin, ribosylzeatin) of eluate was evaporated to dryness in vacuo and the residue was dissolved in 2 ml of 35% aqueous ethanol and chromatographed on an LH-20 column (2.5 diam x 50 cm) developed with 35% aqueous ethanol (Armstrong et al. 1969). The elution profiles from this column procedure

are highly reproducible. The level of i^6Ade and/or derivatives in the column eluates was below that which could be recorded on a spectrophotometer, however figure 1 shows the resolution of a synthetic mixture of four cytokinins (2 mg each) including i^6Ade on this column.

Assay of the LH-20 fractions

1. Bioassay for Cytokinin Activity: the column eluate obtained from the extraction procedure according to procedure 2 (no heating or acidification) was divided into 24 ml fractions. Aliquots of 1 ml from each fraction were added to 25 ml erlenmeyer flasks and the solutions were evaporated to dryness and redissolved in 10 ml of basal medium (Dyson et al. 1970). To each flask was added three pieces of soybean callus, each weighing approximately 0.3 mg. After 35 days, the tissue from each flask was weighed. The results shown in Figure 1 are superimposed over the LH-20 column profile of four authentic cytokinin compounds.

2. Assay by Means of Gas-Liquid Chromatography: the eluates from the column separations of the two extracts obtained from procedures 1 and 2 were divided into six fractions, four of which corresponded to the positions of the four standard cytokinin compounds (Fig. 1) and one immediately before the first peak and one immediately after the last peak. Each fraction was evaporated to dryness in vacuo.

The TMS derivatives were prepared as follows: the residue was dissolved in 10 µl of dry pryridine and 15 µl of BSTFA and the solution was heated for 10 min at 65° in a Teflon covered screw cap reaction vessel. The volume of the reaction mixture decreased and was made up to 20 µl total by addition of BSTFA. An aliquot of this solution was injected immediately into the GLC column. The chromatography was conducted at 210°, gas flow, 35 ml/min (see Alam and Hall, 1971). Results are shown in Figure 2.

We could not detect the presence of zeatin, ribosylzeatin nor i^6Ado in their respective corresponding fractions from the LH-20 columns. We

detected no material corresponding to the known cytokinins in the first and last fractions of the series.

There are some inherent problems in estimating quantitatively the amount of i^6Ade in the extract. N^6(Δ^2-Isopentenyl)adenine elutes relatively early from the GLC column and as a consequence it appears as a shoulder on the pyridine peak that elutes at the beginning of the profile. The sample obtained via extraction procedure 2 contained an unidentified component that had a retention time close to that of i^6Ade.

To obtain a measure of the amount of i^6Ade present originally in the culture we estimated the area under the peaks corresponding to i^6Ade starting from the sloping background as base. The increase in peak area on co-injection of a known amount of i^6Ade served as an internal quantitative standard (it also provided qualitative evidence for the identity of the peak). We estimate from the gas chromatographic data that the yield from one l of the original culture was 12 µg/l when extracted by procedure 1, and 0.4 µg/l when extracted by procedure 2.

The entire extraction procedure was reproducible giving the same GLC profiles. The errors in estimation stem from the high background and the incomplete resolution of the i^6Ade peak. The estimates are in effect upper limits. The important thing is that there appears to be a significant quantitative difference in results between the two extraction procedures.

Extraction of *C. fascians* cultures labeled with [8-^{14}C]-adenine:

Further support for the identity of i^6Ade extracted from the culture and a strict quantitative assessment of the different recoveries obtained via procedures 1 and 2 was achieved using a radioactive labeling technique. A culture of *C. fascians* (one l) was grown up. [8-^{14}C]-Adenine was added in 10 µCi portions 6, 12, 20, and 26 hours after inoculation. Fifty-six hours after inoculation four A_{269} units of i^6Ade was added and the culture was

divided into two equal parts. One part was extracted according to procedure 1 and the other according to procedure 2. The amount of i^6Ade recovered from the LH-20 fractions via each of the two procedures was determined by spectrophotometric analysis. The fractions were then evaporated to dryness and the residues were dissolved in 200 µl of 95% ethanol. The samples were chromatographed on paper in solvent system B. The radioactive profiles of each chromatogram showed a single radioactive spot migrating coincidently with i^6Ade. The amount of radioactivity (adjusted for the percentage recover; 25%) was: procedure 1, 15,200 cpm, procedure 2, 2,740 cpm.

The identity of the radioactive spot as i^6Ade was confirmed by subjecting the recovered material to mild acid hydrolysis (1N HCl, for 15 minutes at 100°). This procedure converts i^6Ade to two unique degradation products (Robins et al. 1967). The hydrolyzed samples contained two radioactive spots migrating coincidently with these hydrolysis products in solvent B.

No radioactive zeatin, ribosyl zeatin, nor i^6Ado was detected in the respective LH-20 fractions from these columns.

Acid Hydrolysis of C. fascians tRNA

In view of the above results, it seemed that heating and subsequent acidification of C. fascians cultures prior to extraction could release i^6Ade from the tRNA. This point was tested further using radioactive tracers. A culture of C. fascians (100 ml) was labeled with 5 µCi portions of [8-^{14}C]-adenine 6, 18, 30 and 42 hr after innoculation. The bacteria were harvested after 60 hr incubation by centrifugation for 10 min at 10,000 g at 4°. The sample was added to 10 g of unlabeled C. fascians cells harvested previously. tRNA was extracted and purified (see methods).

To simulate the extraction conditions of procedure 1 tRNA was dissolved in 2 ml of 0.2 N hydrochloric acid and the solution placed in a water bath, previously heated to 98°. After the solution had warmed to 95° it was allowed to cool for 30 min, under conditions identical with those used for treatment of C. fascians (procedure 1). The solution was neutralized with 0.1 NH_4OH and the reaction mixture was chromatographed on a Celite 545 column (1.9 cm (diam)x50 cm). The first 15 ml of eluate was collected and the contents were chromatographed on Whatman 1 paper in solvent B together with authentic i^6Ade. A single area of radioactivity was found on the chromatogram that coincided with the mobility of i^6Ade. (None of the other derivatives of adenine known to occur in tRNA have a similar Rf value in this solvent system (Hall, 1971)). Since this technique does not give specific labeling of the i^6Ado residues in tRNA we could not quantitate the result, but it shows that i^6Ade is liberated from the tRNA of C. fascians under these extraction conditions.

DISCUSSION

The results reported in this paper bear on the question of whether free i^6Ade is present in cultures of C. fascians and if so at what level? Since the original studies of Thimann and Sachs (1966) and Klämbt et al. (1966) on this problem, new information about the occurrence of i^6Ado in tRNA as well as the chemical properties of i^6Ade and its related compounds has become available.

Klämbt et al. (1966) reported that cultures of C. fascians contain about 10 µg/l of i^6Ade. This value is considerably higher than that which would account for the small amount of cytokinin activity demonstrated by Thimann and Sacks (1966) using an oat-leaf assay. Klämbt et al (1966) suggested that in part the discrepancy between the cytokinin activities extracted by the two procedures were due to differences in the bioassays used. However, there was a greater difference between the two groups estimates of activity than that which could be accounted for by this explanation. This indicates that Klämbt et al (1966) were able to extract more cytokinin from cultures of C. fascians than were Thimann and Sachs (1966). A significant difference between the extraction procedures used by the two groups was that whereas Thimann and Sachs (1966) used a solvent extraction procedure, Klämbt et al (1966) included a mild acid treatment in their procedure. It seems, therefore, that this particular form of extraction released i^6Ade from a bound form.

To clarify this point we analyzed a culture in which the i^6Ade component was labeled with a radioactive marker. This culture was divided in half and the free (unbound) i^6Ade isolated by identical procedures except one included a hot acidification step (procedure 1). This extract yielded 5.5 times as much i^6Ade as procedure 2 which omitted this step.

These data, however, do not furnish absolute values.

To obtain a measure of the amount of i^6Ade present in the culture we carried out a chemical analysis using GLC as the final step. Under these conditions extraction by procedure 1 yielded 12 µg/l of i^6Ade. The relatively small amount of i^6Ade that was being isolated made it difficult to separate it completely from the background. The estimation represents an upper limit. Identification of i^6Ade in the GLC chromatogram was based on the known chromatographic behaviour of this compound in the two previous column systems, partition chromatography on Celite-545 and chromatography on Sephadex LH-20. Finally it should be noted that the GLC technique separates i^6Ade from its related derivatives. The compound isolated from the extract co-chromatographed in the GLC with i^6Ade.

The amount of i^6Ade detected by GLC in the extract obtained by procedure 2 was so little as to be on the threshhold of detectability, certainly much less than that of extraction procedure 1. The difference of 5.5 fold obtained in the radioactive experiment represents a more accurate measure of the difference, so by dividing the 12 µg value by 5.5 it means that the level of i^6Ade in *C. fascians* culture is of the order of 2 µg/l.

The source of the greater quantity of i^6Ade in extract 1 is most likely the tRNA. Matsubara *et al.* (1968) showed that *C. fascians* tRNA contains a cytokinin that they suggest is i^6Ade. Our analysis of this tRNA containing radioactive i^6Ado confirms this conclusion. The glycosyl bond of i^6Ado is susceptible to acid hydrolysis, and cleavage can occur even when the nucleoside is an integral part of the tRNA. In addition to the glycosyl bond cleavage, i^6Ado can undergo a secondary acid catalysed

reaction in which two products are formed, N^6-(3-hydroxy-3-methylbutyl)-adenine and 3H-7,7-dimethyl-7,8,9-trihydropyrimido[2,1-i]purine (Robins et al. (1967). The former product has cytokinin activity (Hall and Srivastava, 1968). If the hydrolysis conditions are favourable, which seems to be the case in procedure 1, the predominant product is the free base, i^6Ade [see Martin and Reese (1968) for comment on this hydrolysis step].

There is the possibility that i^6Ade is bound to a protein and that the heating-acidification treatment results in a more efficient extraction. If this were the case, other cytokinins produced by C. fascians might also be bound in the same manner. Heating-acidification would then liberate all the cytokinins more effectively. After heating and acidification, i^6Ade represented the predominant cytokinin activity in the extract (Klämbt et al., 1966). On the other hand, when the heating and acidification steps were omitted, i^6Ade accounted for only a small portion of the total cytokinin activity (Figure 1). It is doubtful that the additional amount of i^6Ade extracted after heating and acidification can be attributed to liberation from a bound state other than tRNA.

With a clarification of the amount of i^6Ade present in C. fascians cultures, this cytokinin is now placed in perspective with respect to other cytokinin components. Our bioassay results confirm the presence of several cytokinins present in the cultures as shown by Klämbt et al. (1966). According to our results, however, i^6Ade is one of the lesser components accounting for about 20% of the total biological activity.

We have not identified the other cytokinin compounds but the biological activity with respect to column chromatography on both partition and LH-20 column systems coincides with the mobility of i^6Ado, ribosyl zeatin and zeatin. None of these compounds were detected on gas chromatography however.

In the case of zeatin the greater potency means that the absolute amount is less, perhaps below, the threshold of our chemical detection procedure.

In summary, the results of these experiments show that i^6Ade is a normal component of cultures of C. fascians, about 2 µg/l. This cytokinin, moreover, is only one of a group of compounds present that exert cytokinin activity and in terms of total biological activity it represents one of the lesser components.

CAPTIONS

Figure 1. Cytokinin activity of fractions of Sephadex LH-20 column obtained from extract of C. fascians culture (procedure 2). The LH-20 column eluate was divided into 12 ml fractions and a two ml aliquot was taken from each fraction for the assay. The aliquots of fractions 1 - 10, 11 - 16, 41 - 50 and 51 - 60 were pooled for the bioassay. Other aliquots (17 - 40) were assayed in groups of two.

A histogram showing growth response is superimposed over the ultra-violet absorption profile of a separation of four standard cytokinin compounds on the same LH-20 column.

Figure 2. Gas Chromatography of Extracts of C.fascians Cultures

A. Sample obtained by procedure 1. An aliquot of 1.5 µl of a total of 20 µl containing the TMS derivative of the entire sample obtained from the LH-20 column fraction corresponding to i^6Ade. Atenuation,16.

B. Same as in A except with coinjection of 1 µl of a TMS derivative prepared from 20 µg of i^6Ade, final volume of solution 20 µl. Attentuation, 16.

C. Sample obtained by procedure 2. An aliquot of 0.5 µl of a total of 20 µl containing the TMS derivative of the entire sample obtained from the LH-20 column fraction corresponding to i^6Ade. Attenuation, 8.

D. Same as in C except 1.0 µl of aliquot used and sample coinjection with 0.2 µl of the TMS derivative of authentic i^6Ade. (Prepared same as in B). Attenuation, 16.

NOTE: the runs for A & B and C & D were made on different days. The variations in retention times are due to slight variations in operating conditions. Standard samples of the cytokinin compounds were always run each day to verify retention times.

1. Alam, S.N., Hall, R.H.: Separation of the Δ^2 and Δ^3 isomers of $\underline{N}^6$-(Δ^2-isopentenyl)adenosine by gas chromatography and characterization by mass spectroscopy. Anal. Biochem. 40, 424-428 (1971).

2. Armstrong, D.J., Burrows, W.J., Evans, P.K., Skoog, F.: Isolation of cytokinins From tRNA. Biochem. Biophys. Res. Comm. 37, 451-456 (1969).

3. Dyson, W.H., Chen, C.M., Alam, S.N., Hall, R.H., Hong, C.I., Chheda, G.B.: Cytokinin activity of ureidopurine derivatives related to a modified nucleoside found in transfer RNA. Science 170, 38-330 (1970).

4. Girard, M.: Isolation of ribonucleic acids from mammalian cells and animal viruses. Methods in Enzymology 12, (Part A), 581-588 (1967).

5. Hall, R.H.: The modified nucleosides in nucleic acids. Columbia University Press, New York (1971).

6. Hall, R.H., Srivastava, B.I.S.: Cytokinin activity of compounds obtained from soluble RNA. Life Sciences 7 (Part 2), 7-13 (1968).

7. Klämbt, D., Thies, G., Skoog, F.: Isolation of cytokinins from Corynebacterium fascians. Proc. Nat. Acad. Sci. 56, 52-59 (1966)

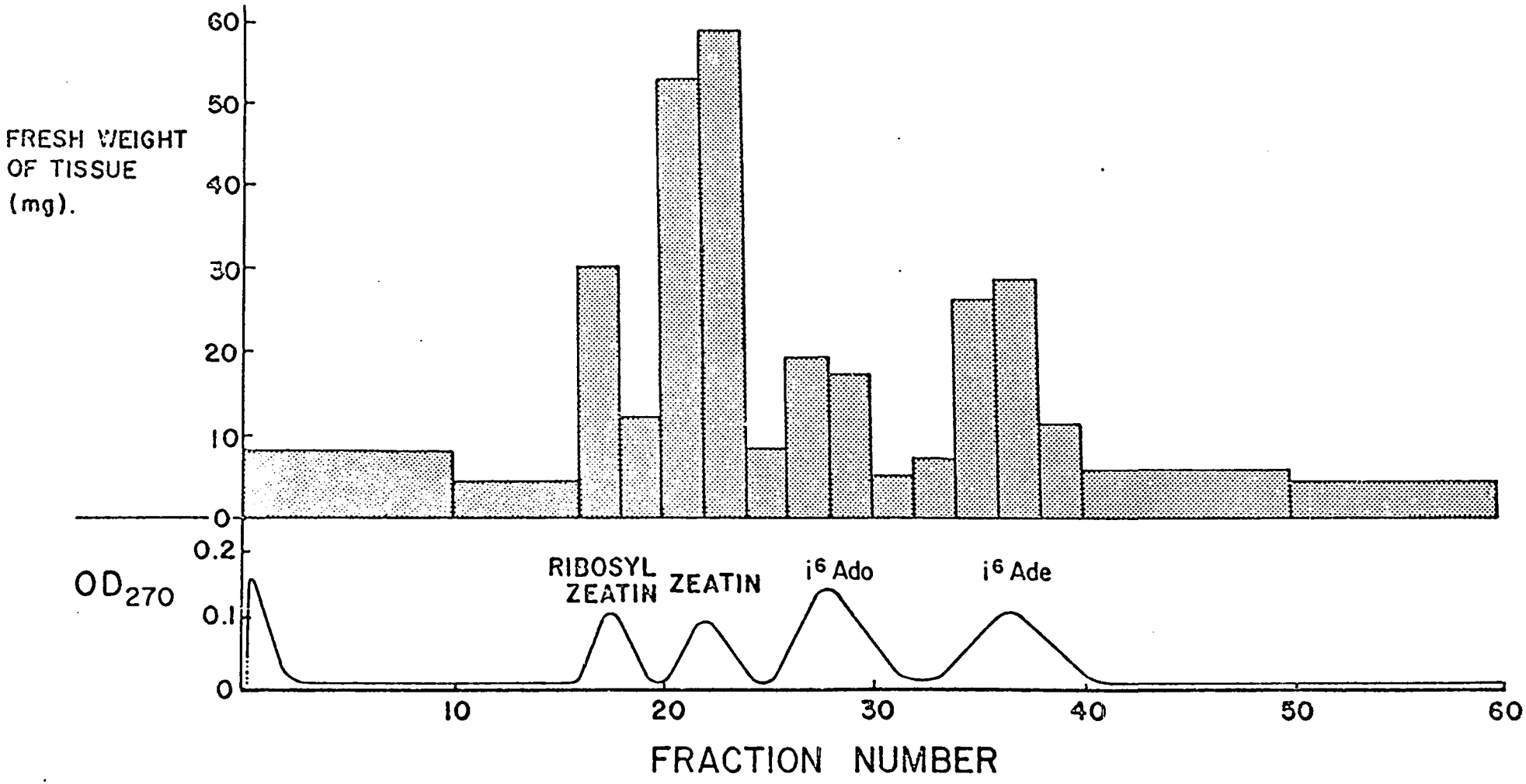
8. Martin, D.M.G., Reese, C.B.: Some aspects of the chemistry of $\underline{N}$(1)- and $\underline{N}$(6)-dimethylallyl derivatives of adenosine and adenine. J. Chem. Soc. (C) 1731-1738 (1968).

9. Matsubara, S., Armstrong, D.J., Skoog, F.: Cytokinins in tRNA of Corynebacterium fascians. Plant Physiol. 43, 451-453 (1968)

10. Robins, M.J., Hall, R.H., Thedford, R.: $\underline{N}^6$-(Δ^2-Isopentenyl)adenosine. A component of the tRNA of yeast and of mammalian tissue, methods of isolation, and characterization. Biochemistry 6, 1837-1848 (1967).

11. Roussaux, J.: Étude préliminaire des modifications induites chez le pois Express Alaska par le Corynebacterium fascians (Tilford) Dowson. Revue Générale de Botanique 72, 21-53 (1965).

12. Thimann, K.V., Sachs, T.: The role of cytokinins in the "fasciation" disease caused by Corynebacterium fascians. Amer. J. Bot. 53, 731-739 (1966).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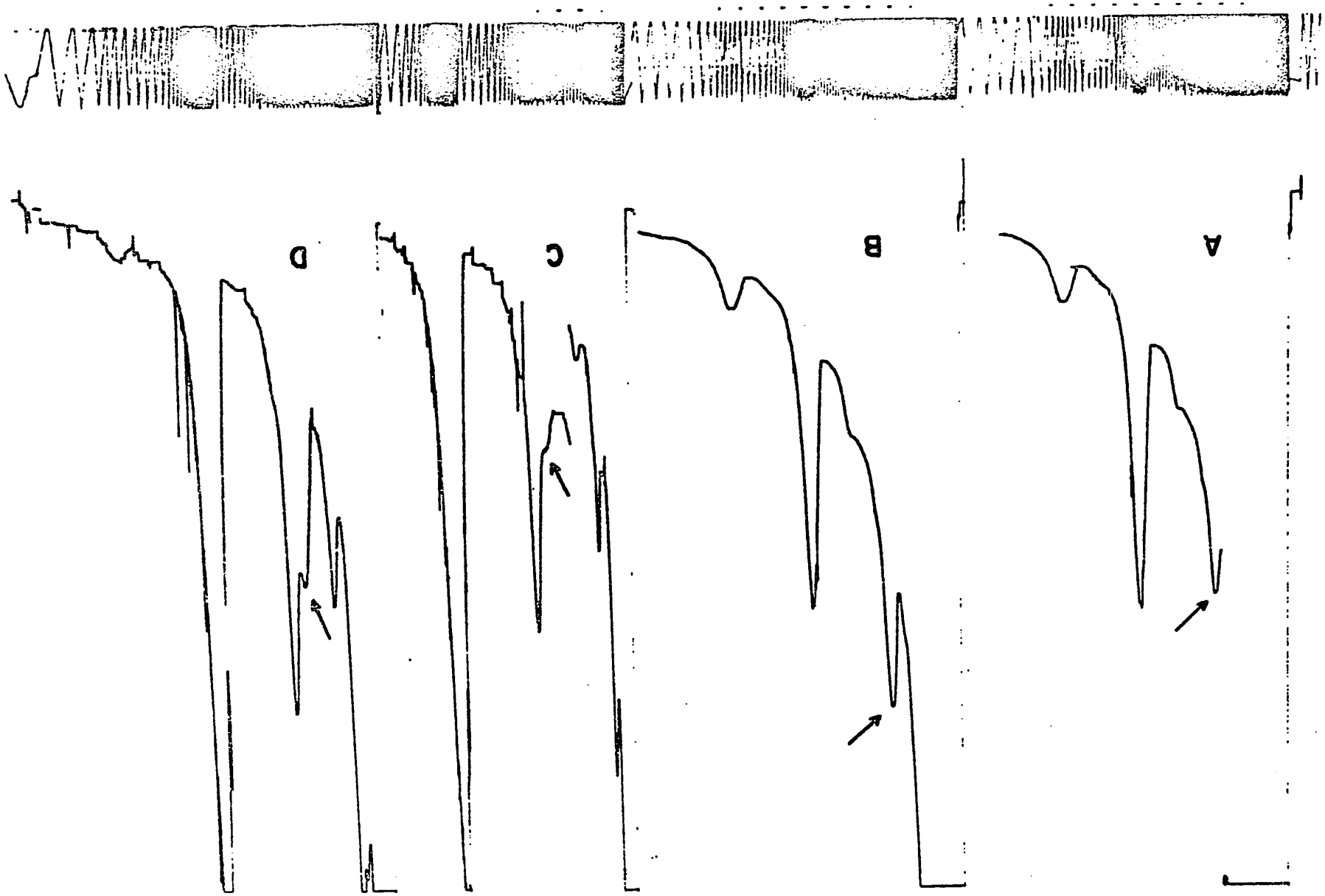
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