Summer 1976

An Investigation of the Nucleotide Cyclic 3'5' Adenosine Monophosphate in Young Hypocotyls of *Pisum sativum* L.

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AN INVESTIGATION OF THE NUCLEOTIDE, CYCLIC 3'5' ADENOSINE MONOPHOSPHATE, IN YOUNG HYPOCOTYLS OF PISUM SATIVUM L.

by

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B.S. June 1974, Old Dominion University

A Thesis Submitted to the Faculty of
Old Dominion University in Partial Fulfillment of the
Requirements for the Degree of

MASTER OF SCIENCE

BIOLOGY

OLD DOMINION UNIVERSITY
August, 1976

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ABSTRACT

AN INVESTIGATION OF THE NUCLEOTIDE, CYCLIC 3'5' ADENOSINE MONOPHOSPHATE, IN YOUNG HYPOCOTYLS OF PISUM SATIVUM L.

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Concentrations of cAMP increased with time in pea hypocotyls assayed at 24 hour intervals over a period of four days. The highest concentration was 20.1 pmoles/mg protein which occurred in 96 hour hypocotyl tips. Ninety-six hour root bodies (dissected region between tip and embryo) achieved a concentration of 8.6 pmoles/mg. Concentrations of cAMP in embryos remained constant around one pmole/mg for three days and values doubled on the fourth day.

Hypocotyls, after germination for 72 hours, were dissected and incubated for various lengths of time in water and 3×10^{-8}M IAA. Tips incubated for two hours showed a tenfold increase in cAMP over water controls. After four hours, cAMP levels in water and IAA incubated tips were comparable. Root bodies incubated in IAA showed a twofold increase in cAMP after two hours and a fourfold increase after four hours. Concentrations of cAMP in embryos remained low but increased slightly with IAA treatment.

Electron micrographs treated to localize adenyl cyclase, the enzyme responsible for cAMP production, revealed discrete membrane bound regions inside cytoplasmic vacuoles which correspond to previously localized enzymes described as acid phosphatases. It remains to be determined whether the specificity of the substrate, adenylyl imido diphosphate (AMP-PNP), used in the present study is such as to exclude all phosphatase activity other than adenyl cyclase or whether the acid phosphatase localized in earlier studies by other investigators is, in fact, an adenyl cyclase. In the differentiated cells of the root cap, adenyl cyclase was localized in discrete areas bound to the smooth endoplasmic reticulum.

ACKNOWLEDGEMENTS

I would like to thank sincerely the members of my graduate committee, Drs. E. F. Stillwell and James H. Yuan, for their helpful criticisms and suggestions throughout the course of this study. Special thanks to Dr. Kneeland Nesius, thesis director, without whose discussion and moral support this study would not have been completed.

I am indebted to Professor Nancy Wade, Dr. Gary Copeland and Dr. Roger Blais for their advice, support and helpful discussions. I am also indebted to Dr. Sanford Vernick for generously permitting me the use of his laboratory and facilities at Eastern Virginia Medical School.

I would also like to express my gratitude to Alicia Hilton for her unflagging encouragement and for typing the manuscript.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Since the first recorded observations of plant behavior by Darwin in his studies of the effect of light on growing stems (Darwin, 1896), there has been an interest in unraveling the complexities of the actions of plant hormones. Much of this interest has been focused on a class of plant hormones generally referred to as auxins. Natural and synthetic auxins have been used commercially as herbicides and in horticultural techniques. They are also important in the developing field of plant tissue culture. Indole acetic acid (IAA) is a naturally occurring auxin, instrumental in mediating a number of processes in plants including the elongation of the walls of young cells. It is the purpose of this research to determine whether cyclic 3'5' adenosine monophosphate (cAMP) plays a role in the action of IAA on young cells of developing pea hypocotyls. Cyclic AMP has been shown to act as a second messenger for a number of hormones in animals (Robison et. al., 1972; Pastan, 1972). The nucleotide occurs in slime molds (Konijn et. al., 1967; Bonner, 1970), as well as the green algae (Amrhein and Filner, 1973). The occurrence of cAMP in vascular plants, however, is in doubt (White et. al., 1973). This research will investigate the role of cAMP in young pea roots to determine: if the nucleotide occurs and in what concentrations with

time in which parts of the hypocotyl; whether application of exogenous IAA results in altered levels of endogenous cAMP; and the location of the enzyme responsible for the production of cAMP.

Cyclic AMP

Experiments leading to the discovery of cAMP by Sutherland and Rall (1958) (Pressman and Sutherland, 1958) were concerned with the effects of catecholamines and glucagon in stimulating glycogenolysis in liver. Action of the hormones at the cell membrane somehow affected the activation of the enzyme, glycogen phosphorylase. The activation was found to be mediated by a kinase which phosphorylated the inactive enzyme. The kinase was found to have active and inactive forms and its activation was dependent on a heat stable factor synthesized from ATP. By purification (Sutherland and Rall, 1958) and independent chemical synthesis of the compound from ATP (Cook et. al., 1957), it was identified as cyclic 3'5' adenosine monophosphate (cAMP). Sutherland demonstrated that cAMP production was induced at the membrane by the action of hormones and that by artificially elevating levels of cAMP, the effects of the hormone could be duplicated. However, he was not able to elucidate the specific mechanism of action of cAMP after the hormone induction. Walsh and Krebs (1966) (Walsh

et. al., 1970) demonstrated that cAMP combined at a regulatory subunit of two phosphorylating protein kinases involved in the rapid breakdown of glycogen in muscle, thus activating the enzymes.

Further elucidation of the system (Sutherland et. al., 1962; Sutherland and Robison, 1968; Walsh et. al., 1968) has led to the well known "second messenger hypothesis" of hormone action. A hormone acts as a first messenger (extracellular effector), binds to active sites on membranes of the target cell where the adenyl cyclase enzyme system is activated, promoting the conversion of ATP to cAMP (the second messenger or intracellular effector). A second enzyme, phosphodiesterase, catalyses the conversion of cAMP to biologically inert 5' adenosine monophosphate (5' AMP) (Butcher and Sutherland, 1962). The cAMP usually serves to activate or inactivate enzymes already present in the cytoplasm (Butcher et. al., 1972). In a great many cases, the action of cAMP has been shown to activate phosphorylating kinases (Walsh et. al., 1970; Greengard and Kuo, 1970; Kuo and Greengard, 1968).

IAA

Indole acetic acid (IAA) is an auxin, a plant hormone which mediates a number of processes including the elongation of cells produced in meristems (Scott, 1972). The hormone is

synthesized from tryptophan in meristematically active tissues (Burnett and Audus, 1964). It is degraded by several processes including oxidation, decarboxylation and conjugation with aspartate (Morris et. al., 1969; Pilet et. al., 1970; Andreae, 1967). Cells undergoing elongation in the root are generally considered to be under the influence of two auxin gradients, the source of one being the high concentration produced by meristems of the epicotyl and transported down the plant axis, and the source of the other being the low levels of auxins produced by root meristems and transported up the plant axis (Burstrom, 1968; Burstrom, 1969). Concentrations of IAA, which stimulate cell elongation in stems, are inhibitory in the root (Burstrom, 1942; Burstrom, 1957). Burstrom (1950, 1969) has pointed out that this inhibitory effect in elongating root cells can be explained by the fact that elongation of cell walls occurs in two phases. The first phase is considered to be a mechanical loosening of the cross linking of cellulose microfibrils in the wall by virtue of the acid nature of IAA. He has shown that even in cells eventually inhibited by high IAA concentrations, the rate of cell wall slippage is proportional to the concentration of IAA. Burstrom noted that roots differ from shoots in that in roots, IAA exhibits a second inhibitory action at the end of cell wall slippage which shortens the duration of the first phase. Synthesis

and insertion of new wall material has been suggested as the mechanism. Other observations of the effect of IAA on plant cells which may or may not be involved in the second phase action of the hormone, but which are reminiscent of the action of cAMP in animal tissues are: certain phosphorylation reactions are affected by IAA in intact cells but not cell free extracts (Bidwell, 1974), and evidence that the effect of IAA on cell elongation is associated with the activation of a preformed enzyme system which mediates cleavage and reformation of wall polymers. IAA stimulates acid phosphatase (Sasaki, 1972); IAA treatment results in lowered ATP/ADP ratios in pea stems (West and Garber, 1967).

The elucidation of the role of cyclic AMP in mammalian tissues was a significant step forward in understanding the mechanism of action of an animal hormone (Butcher et. al., 1972). A corollary to the discovery of the intercellular "second messenger" role of cAMP was the realization that the adenyl cyclase system was ubiquitous in mammalian tissues responsive to external stimuli. The system, however, is not restricted to mammals. The evolution of the role of the compound can be traced back through the lower animal phyla to the prokaryotes (Raymond et. al., 1973; Kuo and Greengard, 1969). The compound is known to occur in two groups of primitive plants. In the slime molds, it acts as

a hormone, inducing the myxameba of Dictyostelium to aggregate (Bonner, 1970; Konijn et. al., 1967). It has also been reported to occur in the green alga, Chlamydomonas, associated with flagellar function (Amrhein and Filner, 1973).

There have been many reports of the occurrence of cAMP in higher plants and the compound has been directly implicated in the actions of several plant hormones. The general opinion, however, is that the evidence is weak (White et. al., 1973; Butcher et. al., 1972), the major objection being directed at methods used for the assay of cAMP (Amrhein, 1974). A general review of the current literature on the subject follows.

LITERATURE REVIEW

Evidence for the occurrence of cyclic 3'5' adenosine monophosphate (cAMP) in higher plants is not conclusive (White et. al., 1973; Butcher et. al., 1972; Raymond et. al., 1973; Amrhein, 1974). Those papers which have attempted to assay plant material for cAMP fall into two general groups, one group employing a chromatographic assay, the second, a competitive binding assay utilizing a protein kinase. The first assay, initiated by the work of Pollard in 1970, employed a technique by which C^{14} labelled adenine is provided to the tissue under study and the rate of incorporation of C^{14} into compounds co-chromatographing with cAMP is determined. It was by this method that Pollard first suggested a role for cAMP in the action of a plant hormone. He concluded that barley seeds treated with Gibberrellic acid (GA) had a higher rate of incorporation of C^{14} into cAMP than control seeds. GA is a plant hormone which induces amylase activity in the endosperm upon germination (Duffus, 1969; Chrispeels and Varner, 1967).

Similar techniques employing co-chromatography of C^{14} adenine compounds with cAMP standards have been used to demonstrate an increased incorporation of cAMP in mustard seedlings germinated in far red light (Janistyn and Drumm, 1972), and several cases of the hormone, indole acetic acid

(IAA), inducing increased C^{14} cAMP in Maize and Avena coleoptiles (Janistyn, 1972; Salomon and Mascarenhas, 1971) and in germinating seeds of lettuce and Bengal gram (Azhar and Murti, 1971). Although this technique permits comparative studies, it does not lend itself to quantification of cAMP values. The technique has been criticized by Amrhein (1974). In attempting to repeat several of the above experiments, he found that the radioactive band which co-chromatographed with cAMP standards could be resolved into several bands by continued chromatography and that none of the more highly resolved bands corresponded exactly to cAMP controls.

A second assay for cAMP involves the use of a protein kinase from mammalian tissue which binds cAMP at a regulatory subunit (Gilman, 1970; Hardman and O'Malley, 1974). Incubation of an unknown amount of cAMP with the kinase and ^{3}HcAMP will result in unlabelled cAMP competing with ^{3}HcAMP in the assay volume. When the protein is separated, lower counts will be bound when greater amounts of unlabelled cAMP are competing in the reaction mixture. With the use of standard curves, the technique permits an accurate quantification of cAMP to the picomole level.

This assay has been widely used in studies of mammalian systems, but only recently applied to studies of higher plants. Brewin and Northcote (1973), utilizing the kinase assay,

determined values of cAMP in Avena coleoptiles ranging from 10 to 200 pmoles/g fresh weight. They also noted an increase of cAMP upon incubation of the tissue in IAA. Several other investigators have utilized the assay on tissues of higher plants and concluded that cAMP is present in detectable levels (Bachofen, 1973; Becker and Ziegler, 1973; Drlica et. al., 1974). Raymond et. al. (1973), using a kinase assay and a bioluminescence assay found relatively high levels of cAMP (30 - 200 pmoles/mg protein) in such diverse plants as tobacco, sycamore, carrot, bramble and lettuce.

In addition to direct assays for cAMP, a body of indirect evidence exists which implicates the compound in the action of a number of plant hormones. The induction of amylase activity in barley endosperm by GA and the possibility of cAMP mediation has been previously mentioned. Exogenous cAMP has been shown to induce amylase synthesis in the absence of GA in barley endosperm (Galsky and Lippincott, 1969). The nucleotide has also been shown to mimic the hormone in the same tissue by inducing ATPase activity and by inducing protease and acid phosphatase activity characteristic of GA treatment (Nickells et. al., 1971).

Barley endosperm is not the only tissue in which cAMP has been shown to mimic GA. In lettuce seedlings, cAMP together with GA has been shown to synergistically enhance

germination (Kamisaka and Masuda, 1971). The nucleotide has also been shown to mimic GA by inducing elongation of hypocotyls in lettuce seedlings (Kamisaka et. al., 1973). A similar effect in Avena coleoptiles has been noted by Hartung (1973). Another GA mediated event which is mimicked by cAMP is the flowering of Lemna under long day conditions (Kessler and Steinberg, 1973; Kessler and Kaplan, 1972).

Similar indirect evidence has linked cAMP to the action of IAA. In addition to the previously mentioned effects, the nucleotide has been reported to enhance elongation in artichoke tubers (Kamisaka and Masuda, 1971b) and to mimic IAA in delaying abscission of Coleus petioles (Salomon and Mascarenhas, 1971b).

MATERIALS AND METHODS

General

The research was conducted in three parts. The first part was intended to determine if cAMP exists in pea hypocotyls and at what concentrations in which part of the plant over a period of four days. At 24 hour intervals tip, root body (area between tip and embryo) and embryo were dissected from twenty seedlings and the batch extracted and assayed for protein and cAMP in duplicate. This experiment was conducted twice. The use of a sensitive competitive protein binding assay for cAMP with phosphodiesterase controls was used in all determinations of cAMP.

The second part of the research attempted to determine whether incubation of plant parts in hormone (IAA) altered the levels of cAMP. Dissected embryos, root bodies and tips from twenty, 72-hour hypocotyls were incubated for 0.5, 2.0 and 4.0 hours in water and 3×10^{-8}M IAA and subsequently extracted and assayed for protein and cAMP in duplicate. This experiment was conducted twice.

The final portion of the research attempted to determine the areas of cAMP production in the plant cell by a histochemical technique for localizing adenyl cyclase, the enzyme responsible for cAMP synthesis. Root tips were incubated in a reaction mixture containing lead nitrate

which precipitates in the presence of inorganic phosphate, a by-product of the synthesis of cAMP from ATP by adenyl cyclase (Reik et. al., 1970). Treated tissue reveals electron dense areas in the cell, visible with electron microscopy, which correspond to sites of adenyl cyclase action. The use of adenylyl imido diphosphate (ICN) in lieu of ATP insured that areas of phosphatase action, other than the cyclase, were not localized (Howell and Whitfield, 1972).

Growth and Harvest

Growth and harvest conditions were essentially the same in all experiments. Pea seeds (Pisum sativum L., early Alaska, Carolina Biological Supply) were washed in a 20% solution of sodium hypochlorite (commercial clorox) for twenty minutes, then washed four times with sterile distilled water. Seeds were then aseptically placed in sterile plastic petri dishes with either sterile distilled water or $3x10^{-8}$M IAA (Carolina Biological Supply) which had been filtered through a 0.22 micron millipore filter. Seeds were then germinated for the appropriate length of time in the dark at 25^{o}C.

At harvest, embryos with intact hypocotyls were removed from the endosperm, washed and placed on absorbent paper. Parts were aseptically dissected. Tips were removed three to four millimeters from the apex. Root bodies were cut

where they joined the embryo. For those parts which required incubation in water or hormone, they were incubated in petri dishes in the dark in much the same manner as described for germination.

Extraction

Dissected parts from twenty seedlings were placed in a small mortar with one ml of cold 0.3M perchloric acid (PCA). After macerating, with 50 to 75 strokes of a pestle, the material was transferred to a centrifuge tube. The mortar and pestle were rinsed with one or two ml of cold PCA (depending on the amount of tissue) and placed in the same centrifuge tube. After centrifugation at 10,000XG for twenty minutes (Lourdes model A-2 Betafuge), the PCA supernatant was removed and stored at -4°C (see section on purification of cAMP). The pellet was resuspended in approximately two ml of 95% ETOH on a vortex mixer and subsequently centrifuged at 10,000XG for ten minutes. After centrifugation, the ETOH supernatant was discarded and the pellet resuspended in two ml of 0.5N NaOH by vortexing briefly. The NaOH fraction was stored at -4°C until the protein determination was done.

Protein Assay

A portion of the two ml NaOH fraction was diluted one to nine in NaOH and assayed in duplicate for protein by the method of Lowry et. al. (1951). Four reagents were used:

A - 2% Na_2CO_3 in 0.1N NaOH

B - 0.5% $CuSO_4 \cdot 5H_2O$ in 1% sodium potassium tartrate

C - 5 ml of reagent A plus 1 ml of reagent B (discarded after thirty minutes)

E - 1:1 diluted Folin's reagent, 1N in acid

To protein in 0.5 ml, two ml of reagent C was added and permitted to stand at room temperature for 10 to 15 minutes. 0.25 ml of reagent E was then added and the tube was mixed rapidly. Absorbance was read at 500 nanometers at exactly 30 minutes on a Coleman model 6/20 spectrophotometer. The standard was composed of two serial twofold dilutions of a 1 mg/ml solution of bovine serum albumin.

Purification of cAMP

In order to purify partially the cAMP contained in the PCA supernatant, a Dowex 50 H^+ (BioRad AG50 WX4) 100-200 mesh column was utilized (Hardman and O'Malley, 1974). The dimensions were 1 x 7.5 centimeters with a head of 14 - 15 centimeters. Elution with 0.1N HCl yielded a rate of 0.8 ml/min from the column. In order to calibrate the column, to one ml of a PCA extract from pea roots was added 5 microliters of ^{3}HcAMP and the mixture was applied to the top of the column. The 18th through 40th ml from the column contained 97% of the ^{3}HcAMP (see Fig. 1). This 22 ml fraction was collected for each purified sample, lyophilized to dryness (RFS, Incorporated) and brought back up in

0.5 ml of 0.05M Tris hydroxyamino methane. The HCl in the lyophilized portion yielded a final solution buffered at about pH 7.5.

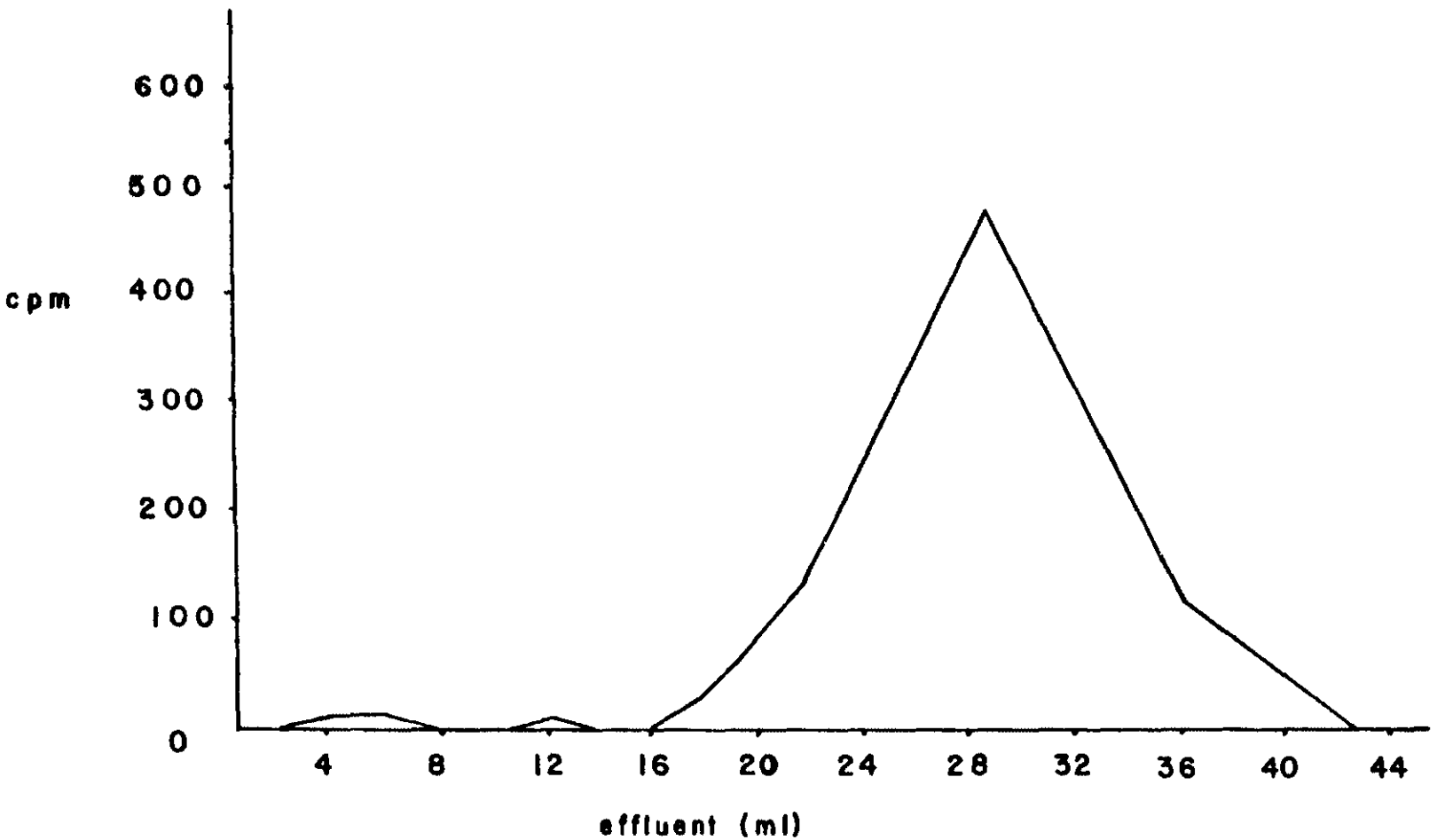
In order to equilibrate the Dowex resin, it was washed twice with two bed volumes of 0.5N NaOH, twice with distilled H_2O, twice with 2N HCl, twice with distilled H_2O, and once with 0.1N HCl. It was stored refrigerated in 0.1N HCl.

cAMP Assay

Fifty microliter aliquots of the Tris samples were assayed in duplicate for cAMP using Amersham/Searle Cyclic AMP Assay Kit TRK. 432. The assay was conducted in a total volume of 200 microliters. Each reaction mixture contained: cAMP (50 ul of unknown or standard), ^{3}HcAMP (50 ul), protein kinase (100 ul), Tris-EDTA buffer. The reaction was sustained for two hours at $0^{\circ}C$. The reaction was stopped by the addition of a charcoal adsorbent which binds the free cAMP. After centrifugation, the supernatants were counted in PCS on a Beckman liquid scintillation counter.

As a control, 50 microliters of the Tris samples were incubated with a cAMP specific phosphodiesterase (Calbiochem) for thirty minutes at room temperature. The reaction was terminated by placing the tubes in a boiling water bath for five minutes. The samples were then assayed for binding activity. Binding remaining after phosphodiesterase treatment represents compounds other than cAMP which are capable of binding with the kinase (cGMP, ATP).

Figure 1. Calibration of Dowex H^+ column with ^{3}HcAMP. Ninety-seven percent of the cAMP was recovered between the 18th and 40th ml from the column.


600
500
400
300
200
100
0
cpm
4
8
12
16
20
24
28
32
36
40
44
effluent (ml)

Average cpm's (cx) less a blank were divided into the average cpm's in the control tube (co) which had no unlabelled cAMP competing with ^{3}HcAMP for sites on the protein kinase. The value for co/cx was then read off a standard curve obtained by running samples of known concentrations of cAMP. After determination of the cAMP contained in each 50 microliter aliquot, values for binding after phosphodiesterase treatment were subtracted and the remainder multiplied by dilution factors to yield the cAMP values expressed in the results.

Localization of Adenyl Cyclase (Reik et. al., 1970; Howell and Whitfield, 1972; Wagner and Bitensky, 1970)

Tips dissected from 48 hour pea hypocotyls were macerated in 1% glutaraldehyde in 0.05M cacodylate nitrate buffer (pH 7.4) and 4% glucose and fixed for one hour. They were then subjected to one of the following treatments for one hour, shaking:

A) Incubation in 80 mM tris maleate (pH 7.4) plus 4% glucose

B) Incubation in 80 mM tris maleate (pH 7.4), 4% glucose, 4.8 mM $PbNO_3$

C) Incubation in 80 mM tris maleate (pH 7.4), 4% glucose, 4.8 mM $PbNO_3$, 2 mM theophylline, 4 mM $MgSO_4$, 0.5 mM AMP-PNP.

All tissues were then fixed in 4% glutaraldehyde in tris maleate (three hours), rinsed five times in buffer, post fixed in 1% osmium tetroxide for one hour, washed and dehydrated in successive solutions of 50% ETOH, 70% ETOH in 0.5% uranyl acetate, 85%, 95% and 100% ETOH. Each dehydration was 10 to 15 minutes with the exception of the 70% ETOH and uranyl acetate which was left overnight. Tissues were embedded in spur low. Thin sections were stained with uranyl acetate, then lead citrate. Micrographs were taken on a Phillips Transmission Electron Microscope, Model 301.

RESULTS

Part One

Average protein and cAMP for tips, root bodies and embryos of twenty hypocotyls germinated in water and assayed at 24 hour intervals over four days are given in Table 1. In general, the tip, root body and embryo demonstrated stable protein values over three days with a slight decrease after four days. Nutrients provided by the endosperm after three days are apparently not sufficient to support continued growth. Some fluctuation in protein values are expected, as twenty hypocotyls were dissected at each time interval without regard to weight.

Tips demonstrated stable protein values between 1.0 and 1.3 mg over the first three days. After 96 hours, protein dropped to 0.7 mg. This drop at 96 hours is partly responsible for inflating cAMP values when expressed in terms of protein. Total cAMP (Table 1) at 96 hours shows that cAMP values approximately doubled between 72 and 96 hours, thus the increase of pmoles cAMP per mg protein (Fig. 2) is not due entirely to the decrease in protein.

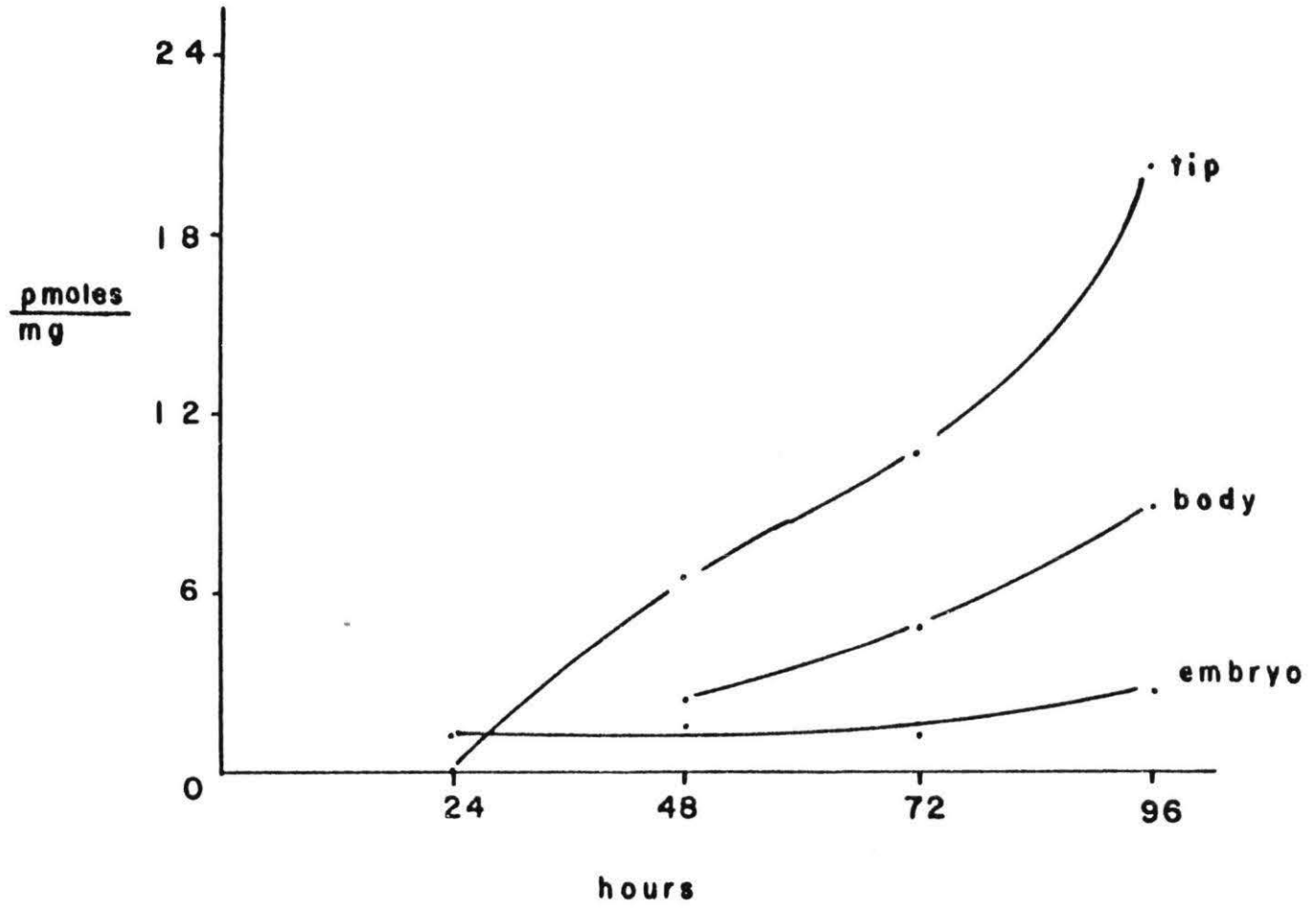
No figures for protein and cAMP in root bodies were determined for 24 hours because hypocotyls had not emerged enough to dissect them. Values for protein in the root bodies increased from 2.8 mg at 48 hours to 3.4 mg at 72 hours. This is the pattern which would be expected, a gain in protein by

Table 1. Total protein and cAMP in hypocotyl tips, root bodies and embryos at 24 hour intervals over a period of four days.

Plant Part	Hours before Dissection	Protein (mg)	cAMP (pmoles)*
Hypocotyl tips			
	24	1.1	0
	48	1.3	6.9
	72	1.0	5.8
	96	0.7	12.1
Root bodies			
	24	--	--
	48	2.8	5.5
	72	3.4	17.0
	96	2.3	17.0
Embryo			
	24	8.0	8.3
	48	6.7	8.5
	72	7.9	7.8
	96	6.4	14.3

*Cyclic AMP values are corrected for binding which remained after phosphodiesterase treatment.

Figure 2. Picomoles cAMP per mg protein in dissected parts of 20 hypocotyls assayed at 24 hour intervals over four days. Each value is corrected for binding remaining after phosphodiesterase treatment and is an average of results from two separate experiments.


24
18
12
6
0
pmoles
mg
tip
body
embryo
24
48
72
96
hours

the root body at the expense of the embryo and cotyledons with protein values for the tips remaining approximately the same. At 96 hours, average protein in the root bodies decreased to 2.3 mg, lending further support to the observation that maximum growth under these conditions occurred at 72 hours. Total cAMP in root bodies increased from 5.5 picomoles at 48 hours to 17.0 picomoles at 72 and 96 hours. These were the highest values of total cAMP recorded for this part of the experiment. When the values are expressed in terms of protein, however, (Fig. 2) they are second in magnitude to the tips owing primarily to the fact that root bodies had two to three times the amount of protein than that occurring in the tips. Most of the 96 hour increase in pmoles cAMP per mg protein in root bodies can be attributed to a decrease in protein at that time.

Values of protein determined for the embryo portion of hypocotyls showed the greatest fluctuation over the four day period (Table 1). Embryos by far represented the greatest amount of tissue in terms of weight (half again as much weight as occurred in root bodies and twenty times the average weight of tips). Since twenty parts were dissected without regard to weight, the fluctuation in protein for twenty embryos can be partly accounted for on this basis. The relationship of protein in embryos with time is generally a straight line slightly decreasing over four days, the lowest value for

protein (6.4 mg) being recorded on the fourth day. Total cAMP remained relatively constant, eight picomoles for the first three days after which the value increased to 14.3 picomoles at 96 hours. Four days roughly corresponds to emergence of the epicotyls from the embryos. Since no attempt was made at 96 hours to remove epicotyls, they were assayed as part of the embryo tissue. The sharp increase of cAMP at four days after three days of relatively low concentrations may be a result of processes initiated with epicotyl emergence. The increase in protein synthesis associated with meristematic activity of the epicotyl is not reflected in the protein values recorded for the embryo at 96 hours. This may be due to growth conditions or weight considerations already mentioned.

The highest concentration of cAMP relative to protein occurred in the tips (Fig. 2). The amount of tissue extracted for assay was small (in the range of 20 mg wet weight). Protein values for tips were also relatively low while cAMP values ranged from 0 to 12.1 picomoles. It should be mentioned that dissection of the tips was made in such a manner as to include most of the region of actively elongating cells. Root bodies contained mostly differentiated tissue.

The lowest concentration of cAMP relative to protein occurred in the embryos where it remained relatively constant, around one picomole per mg protein, for three days and

Total cAMP and cAMP relative to protein increased in all plant parts over the four day period. Values for tips approximately doubled every 24 hours (0, 6.7, 10.8 and 20.1 pmoles/mg). Values for the root body also demonstrated a doubling from a lower initial level (2.3, 4.7 and 8.6 pmoles/mg). All values for cAMP are low when compared to values commonly occurring in animal tissues (Steiner et. al., 1972).

Part Two

Average protein and cAMP values determined for twenty 72 hour hypocotyl tips, root bodies and embryos incubated for one half, two and four hours in water and IAA are given in Table 2.

Hypocotyl tips were the only part incubated in water which showed a decrease in protein over the four hour period. Initial levels after half an hour were 1.1 mg, a figure in close agreement with values for 72 hour tips from experiment one. After two hours of incubation, average protein dropped to 0.9 mg. After four hours, 0.5 mg of protein was recorded for twenty tips. Protein for all parts incubated in water dropped initially. Protein in root bodies and embryos increased after the initial fall. The drop in protein is probably due to damage inflicted on the tissue by dissection and the subsequent placement of cut tissues into a hypotonic

Table 2. Total protein and cAMP in hypocotyl tips, root bodies, and embryos of 72 hour pea hypocotyls incubated for various lengths of time in either sterile distilled water or sterile $3x10^{-8}$M IAA.

Plant Part	Hours Incubated	mg Protein (H_2O)	mg Protein (IAA)	pmoles cAMP* (H_2O)	pmoles cAMP* (IAA)
Hypocotyl tips	0.5	1.1	0.7	6.0	6.0
	2.0	0.9	0.5	1.8	11.4
	4.0	0.5	0.5	4.1	4.1
Root bodies	0.5	2.4	3.0	2.0	2.9
	2.0	3.1	2.4	3.3	4.0
	4.0	3.1	1.5	2.2	5.7
Embryos	0.5	6.6	6.8	7.4	6.0
	2.0	7.9	5.5	6.9	9.5
	4.0	8.1	5.1	5.7	10.1

*cAMP values are corrected for binding which remained after phosphodiesterase treatment.

medium like distilled water. Twenty tips weighed very little (approximately 20 mg, compared to 350 mg and 600 mg for root bodies and embryos respectively). Cutting of tips damaged a large percentage of tissue. The low weight of tip tissue also makes it more susceptible to diffusion of materials into the water. The cut surface of a tip represents a larger percentage of the total surface area than cut regions of embryos and root bodies. Other factors which may have contributed to the failure of tips to recover protein synthesis are the known secretory activity of meristematic and root cap cells, and the fact that IAA is believed to be produced in tips and can inhibit protein synthesis (Bidwell, 1974; Key and Shannon, 1974).

Protein in those tips incubated in IAA decreased from 1.0 mg protein at 0 time (from Part One) to 0.7 mg protein after half an hour and dropped again at two hours to 0.5 mg protein. Tips assayed at four hours also had 0.5 mg protein. Protein decreased in all hypocotyl parts incubated in IAA. This observation is consistent with the known inhibitory effects on roots of IAA at the concentration employed.

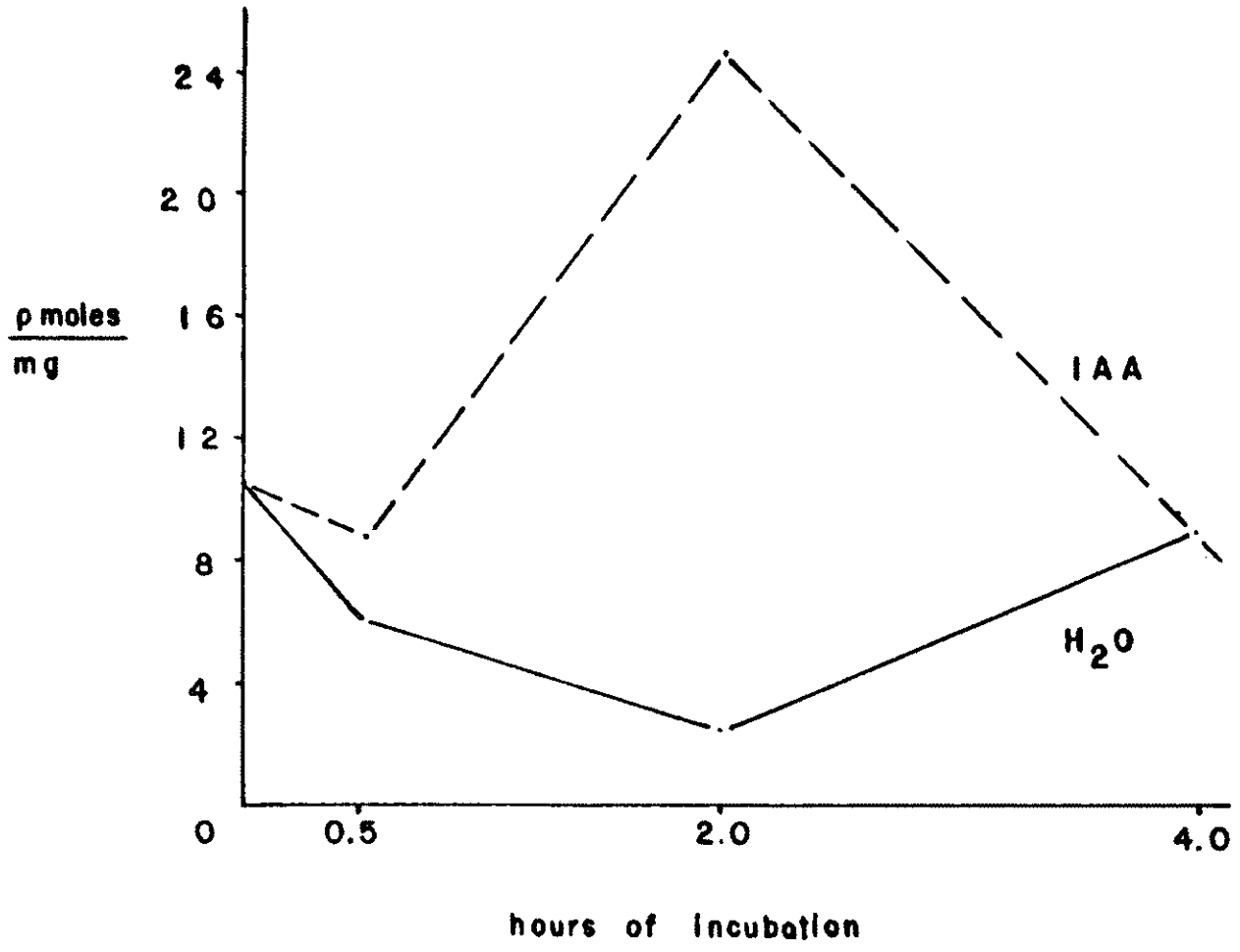
Cyclic AMP in twenty tips incubated for half an hour in water averaged 6.0 picomoles which is in close agreement with 5.8 picomoles recorded for 72 hour tips in Part One. After two hours in water, cAMP levels fell to 1.8 picomoles which suggests that the nucleotide may be leaching out of the tissue. After four hours, concentrations of cAMP

averaged 4.1 picomoles, indicating a slight recovery by the tips of the ability to retain and/or synthesize the compound.

Tips incubated in IAA demonstrated an elevation of total cAMP after two hours. Initially, cAMP values for tips in IAA were comparable to values for tips incubated in water, as are values after four hours. The elevation of cAMP levels in tips by exogenous IAA appeared to occur in a relatively restricted period of time. The fact that a half hour incubation is not sufficient for IAA to affect cAMP values may be due to difficulties in permeability of the tissue to IAA. Return of cAMP levels after four hours to levels comparable to those occurring in tips incubated in water may be a result of the metabolism and/or inactivation of IAA. These data do not preclude the possibility of indirect effects of IAA on cAMP synthesis.

A comparison of cAMP relative to protein in tips incubated in water and IAA is given in Figure 3. Although protein of tips incubated in water decreased dramatically over two hours, it does not decrease enough to cause cAMP expressed in terms of protein to increase. Only after four hours does the tissue show a partial recovery of pmoles cAMP/mg protein. Tips incubated in IAA show a slight decrease of values, then a sharp increase at two hours to 24.9 pmoles/mg. This is the highest level recorded for the nucleotide and represents a tenfold increase over levels found in

Figure 3. Picomoles cAMP per mg protein of 72 hour hypocotyl tips incubated in $3x10^{-8}$M IAA or sterile distilled water. Each value is corrected for binding remaining after phosphodiesterase treatment and is an average of results from two separate experiments.

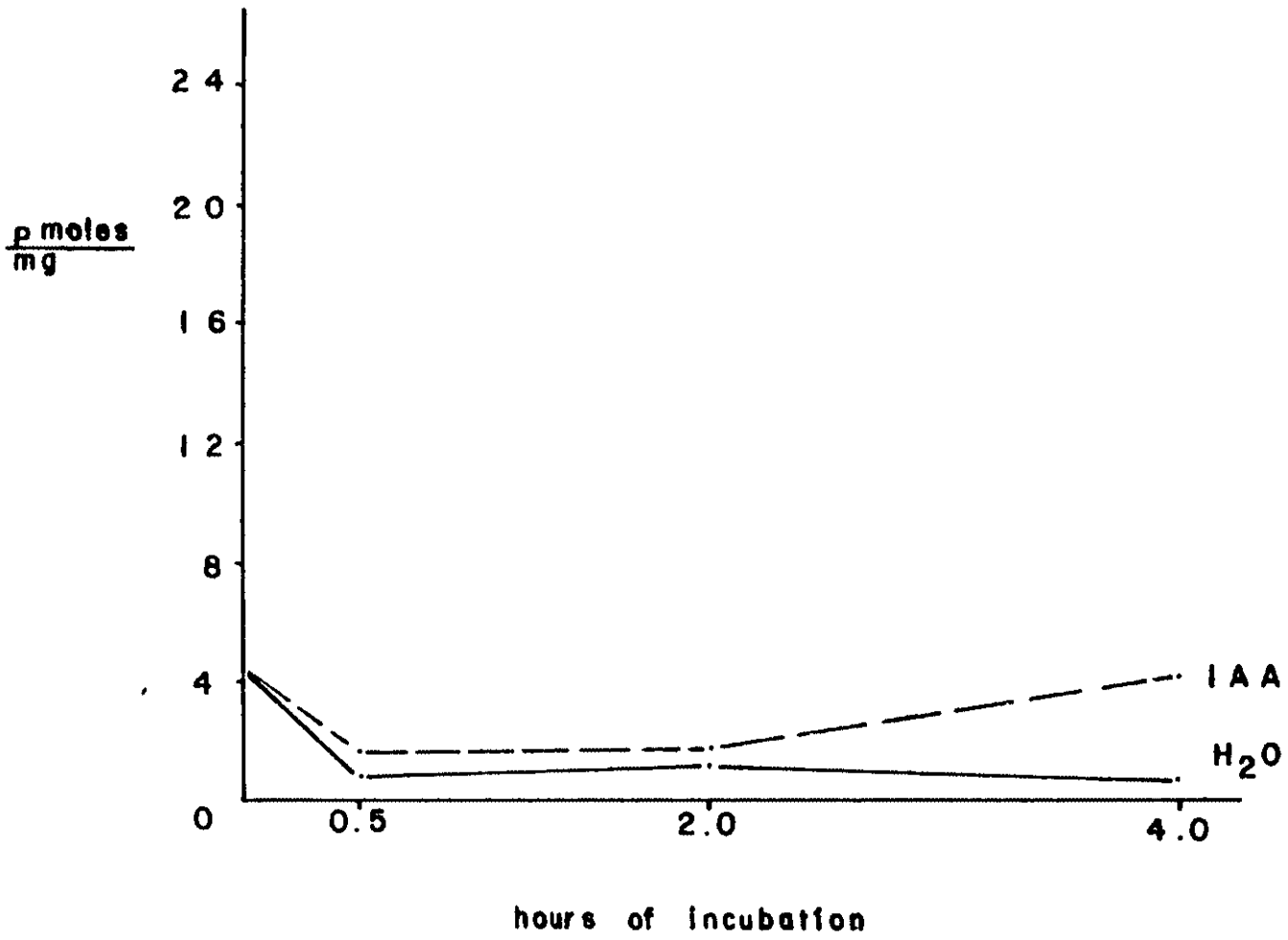

24
20
16
12
8
4
0
p moles
mg
IAA
H2O
0
0.5
2.0
4.0
hours of Incubation

water controls. After four hours, cAMP values for tips incubated in IAA are comparable to water controls.

Values for protein in root bodies and embryos incubated in IAA and H_2O are also given in Table 2. Protein in both tissues increased with incubation in water. Root bodies went from 2.4 mg at half an hour to 3.1 mg after two hours and four hours. Protein in embryos increased from 7.9 mg after two hours. At four hours, average protein in twenty embryos incubated in water was 8.1 mg. The same tissues incubated in IAA demonstrated a steady decrease in protein concentrations. Root bodies went from 3.0 mg protein after half an hour to 2.4 mg at two hours and 1.5 mg after four hours of incubation. Embryos steadily decreased from 6.8 mg protein after half an hour to a value of 5.1 mg after four hours.

cAMP in root bodies incubated in water ranged from 2.0 to 3.3 picomoles and showed no pattern of change. Those root bodies incubated in IAA, however, demonstrated a twofold increase of total cAMP over the four hour incubation. Values for cAMP relative to protein in root bodies incubated in IAA and water are given in Figure 4. In tissues incubated in water, cAMP decreased from 4.7 pmoles/mg at 0 time to 0.9 pmoles/mg at half an hour. Values did not significantly change with further incubation. Incubation of the same tissue

Figure 4. Picomoles cAMP per mg protein of 72 hour root bodies incubated in $3x10^{-8}$M IAA or sterile distilled water. Each value is corrected for binding remaining after phosphodiesterase treatment and is an average of results from two separate experiments.


p moles / mg
24
20
16
12
8
4
0
0.5
2.0
4.0
IAA
H2O
hours of incubation

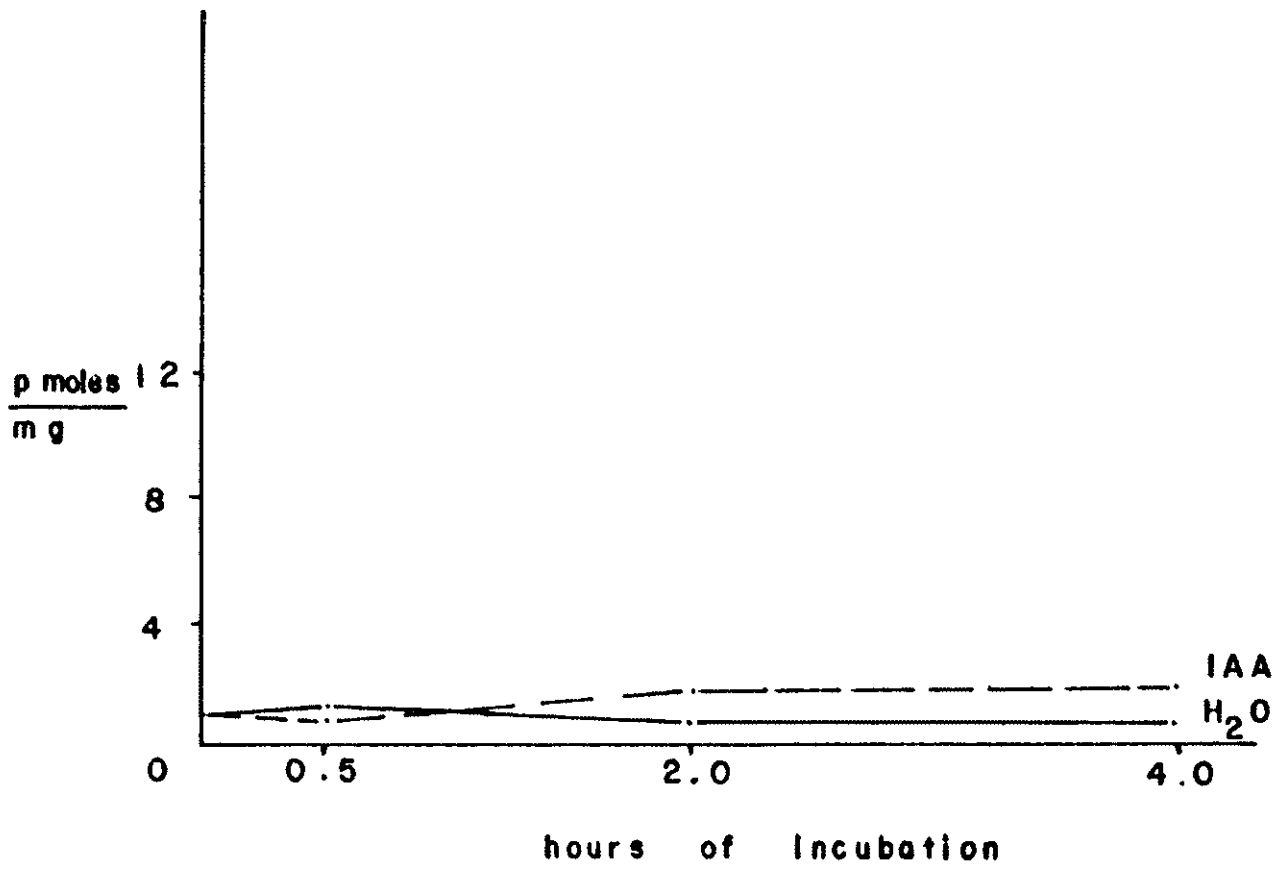
in IAA also demonstrated a drop in pmoles/mg protein after half an hour. After four hours of incubation, levels of cAMP were restored to the initial value of 4.1 pmoles/mg. The dramatic rise in cAMP values after two hours in tips incubated in IAA was not observed in root bodies incubated in IAA. The increase of cAMP expressed in terms of protein is due to both decreases in protein with incubation and increases in total cAMP.

Values for cAMP relative to protein in embryos incubated in IAA and water are given in Figure 5. Embryos incubated in water maintained values of cAMP of 1.0 pmoles/mg for the entire period of incubation. IAA incubation resulted in doubling at two hours to 1.9 pmoles/mg, which was unchanged after four hours of incubation (2.0 pmoles/mg).

All plant parts assayed demonstrated an increase of cAMP relative to protein when incubated in IAA and compared to tissue incubated in water (Figures 3, 4 and 5). The tissues most sensitive to IAA treatment were hypocotyl tips. This sensitivity was restricted to a limited time frame between one half and four hours of incubation in IAA.

Tips and root bodies incubated in water and IAA demonstrated an initial drop in cAMP relative to protein upon incubation. This drop was not observed in embryos.

Figure 5. Picomoles cAMP per mg protein of 72 hour embryos incubated in $3x10^{-8}$M IAA or sterile distilled water. Each value is corrected for binding remaining after phosphodiesterase treatment and is an average of results from two separate experiments.


p moles / m g
12
8
4
0
0.5
2.0
4.0
IAA
H_2O
hours of Incubation

Part Three

Figure 6 is an electron micrograph of an undifferentiated cell from hypocotyl tips incubated in buffer and glucose (treatment A). It is presented as a lead free control.

Figure 7 is a micrograph of similar cells treated with lead nitrate but not provided with substrate (treatment B). Very light precipitation products can be seen in vacuoles in the upper left hand corner. Since precipitation of lead by inorganic phosphates was dependent on endogenous ATP, the formation of small amounts of lead precipitation was not unexpected.

Figure 8 is a micrograph of cells treated with lead nitrate and provided with AMP-PNP (treatment C). Dense precipitates of the lead have formed in cytoplasmic vacuoles. Each vacuole is characterized by a large acentrically located precipitate of lead and by small complexes located in discrete units around the internal edge of the membrane.

All of the cells have relatively thin walls, abundant cytoplasmic vacuoles and large nucleii all of which are characteristic of immature cells. The photographs indicate that adenyl cyclase occurs membrane bound inside cytoplasmic vacuoles.

Figure 9 is an enlargement of the cytoplasm of a differentiated cell of the root cap. Lead complexes have formed in distinctive units attached to the smooth endo plasmic reticulum.

Figure 6. Cells of pea hypocotyl tips incubated in 80mM tris maleate (pH 7.4) and 4% glucose (Treatment A). Tris maleate buffered osmium tetroxide fixation. Sections were stained with uranyl acetate and lead citrate. Magnification is 22,800 X.

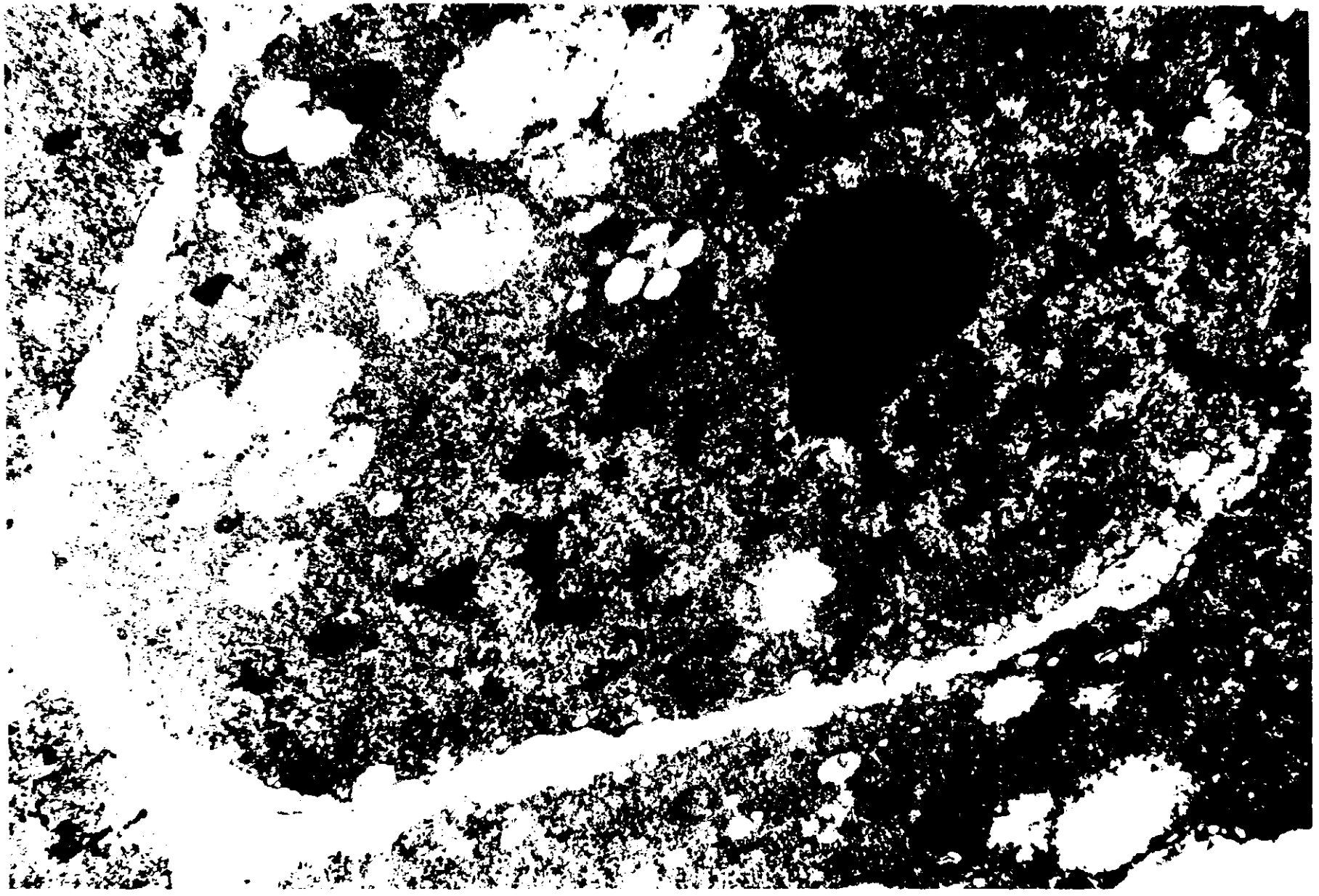


Figure 7. Cells of pea hypocotyl tips incubated in 80mM tris maleate (pH 7.4), 4% glucose and 4.8mM $PbNO_3$ (Treatment B). Tris maleate buffered osmium tetroxide fixation. Sections were stained with uranyl acetate and lead citrate. Magnification is 14,440 X.

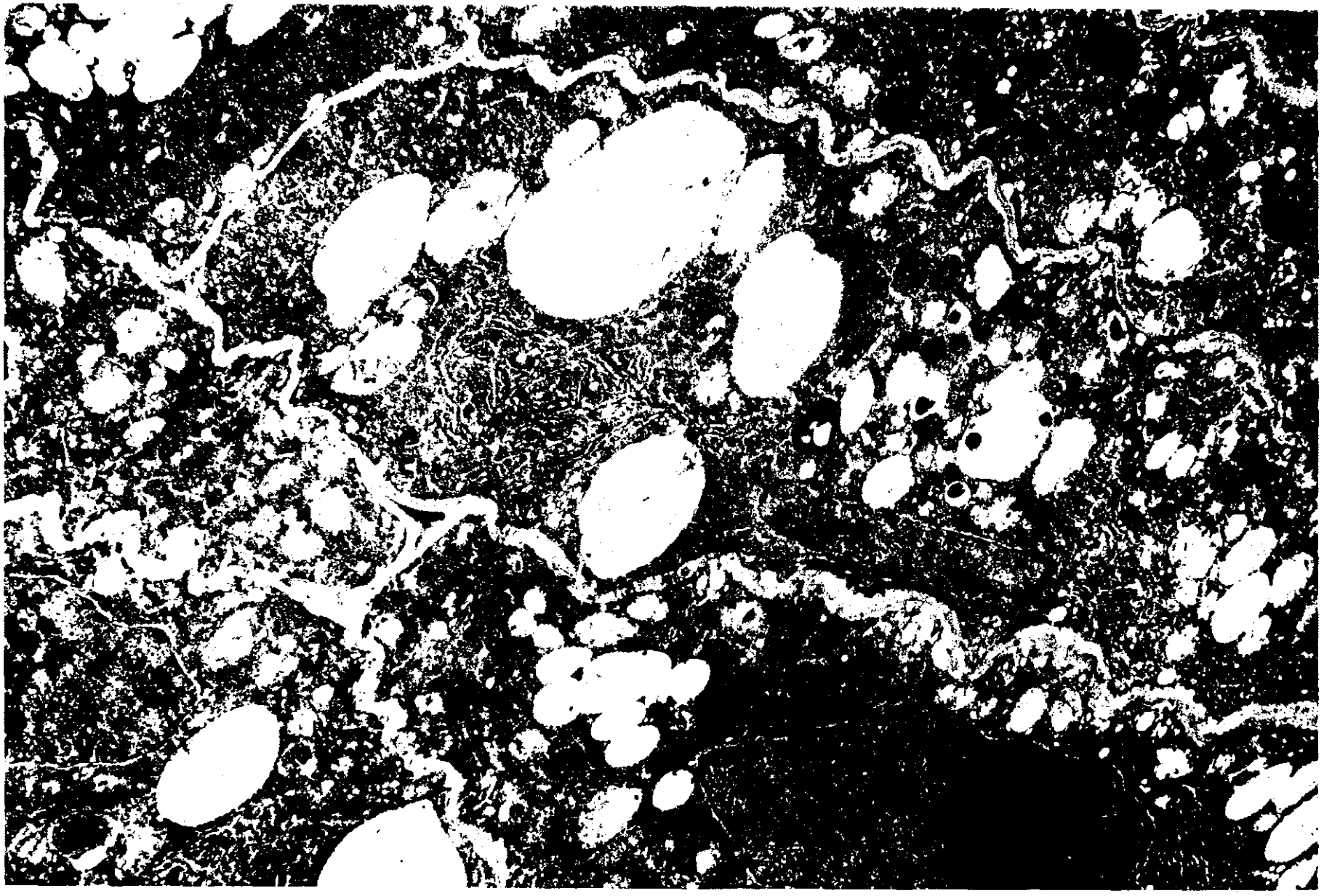


Figure 8. Cells of pea hypocotyl tips incubated in 80mM tris maleate (pH 7.4), 4% glucose, 4.8mM $PbNO_3$, 2mM theophylline, 4mM $MgSO_4$, 0.5mM AMP-PNP (Treatment C). Tris maleate buffered osmium tetroxide fixation. Sections were stained with uranyl acetate and lead citrate. Magnification is 27,300 X.

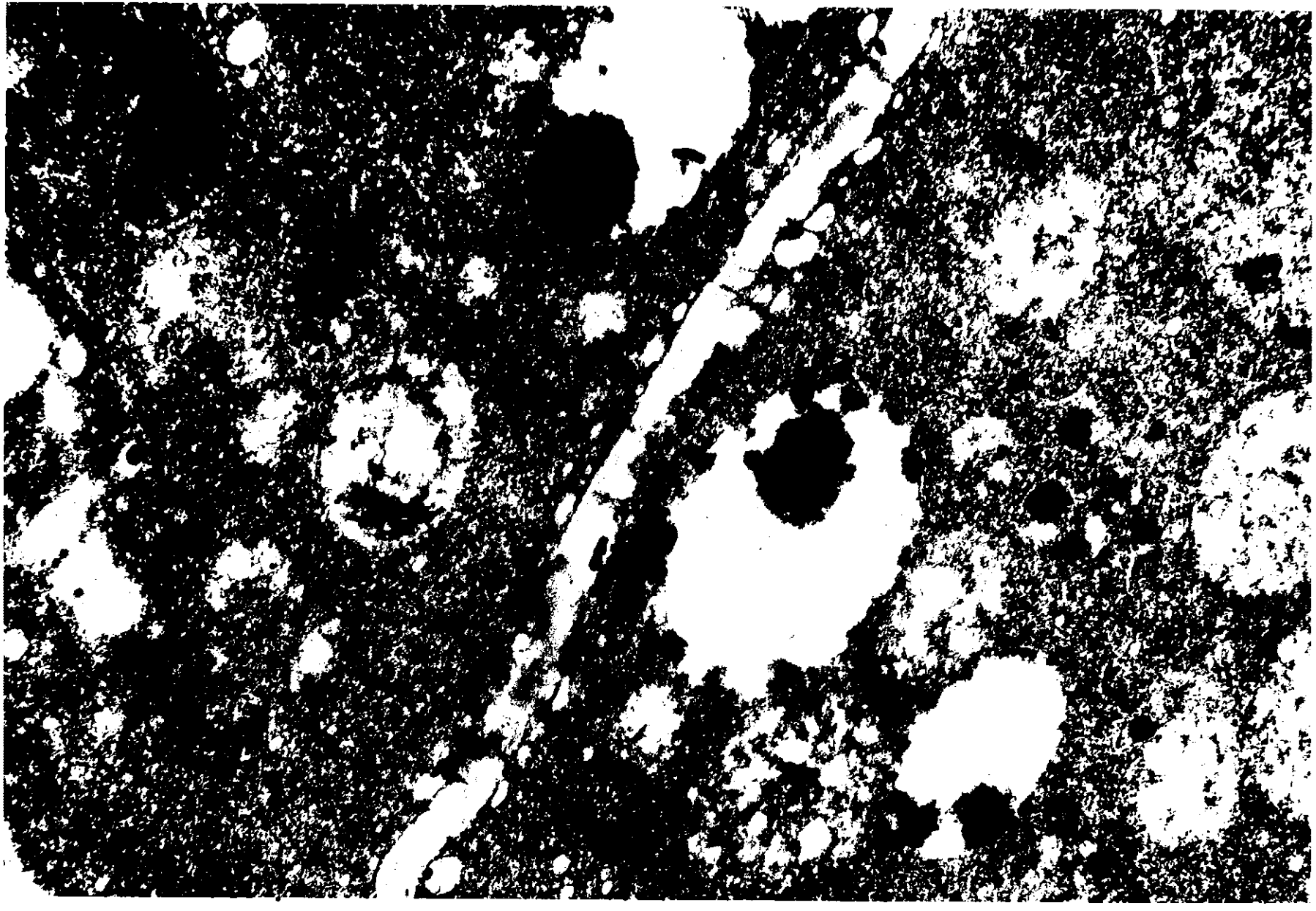


Figure 9. Root cap cell of pea hypocotyl incubated in 80mM tris maleate (pH 7.4), 4% glucose, 4.8mM $PbNO_3$, 2mM theophylline, 4mM $MgSO_4$, 0.5mM AMP-PNP (Treatment C). Tris maleate buffered osmium tetroxide fixation. Sections were stained with uranyl acetate and lead citrate. Magnification is 45,000 X.

DISCUSSION

General

Detectable levels of cAMP are found in all tissues assayed except tips dissected at 24 hours (Fig. 2). Tips at 24 hours were just beginning to emerge from the embryos. Repeated assay revealed no detectable levels of cAMP. These data, then, do not support the argument by some that the nucleotide plays a role in controlling mitosis in plant cells (Wood et. al., 1972; Wood and Braun, 1973; Drlica et. al., 1974). Raised levels of cAMP have been associated with a promotion of cell division in cultured plant tissue. Embryos, after 96 hours in Part One (Fig. 2), demonstrated a doubling of cAMP which appears to be correlated with epicotyl emergence. The increase in cAMP, in light of data from hypocotyl tips, is most likely due to processes occurring in the embryo portion of that tissue extract, rather than with the meristematic activity of the epicotyl. Dissection and assay of epicotyls over a period of time would determine whether this is the case. When expressed in terms of protein, cAMP in all tissues increased with time over four days, in spite of indications that growth conditions were unfavorable after three days. Values for pmoles cAMP/mg protein were highest in the undifferentiated tissue of the tip and lowest in the embryo.

Dissected hypocotyl tissue incubated in an inhibitory concentration of IAA demonstrated a twofold increase in cAMP when compared to water controls, with the exception of hypocotyl tips. Tips (which contained meristematic and undifferentiated cells) demonstrated a tenfold increase in cAMP upon incubation in IAA. The increase was observed after two hours of incubation but not after one half or four hours of incubation. Burstrom (1969) has noted that two hours is the time period in which the inhibitory phase of IAA is usually manifested in responsive tissue. If cAMP is in fact involved in the inhibitory phase of IAA action, several mechanisms are possible. Cytoplasmic vacuoles of immature plant cells are known to contain a variety of enzymes which cAMP may serve to activate. Among the contents of vacuoles may be synthetic enzymes responsible for production of components involved in wall stabilization. Fusion of the many small vacuoles associated with immature cells accompanies the elongation process when the large central sap vacuole characteristic of mature cells is formed (Scandalios, 1967; Evans, 1974). The possibility of cAMP involvement in the initiation of vacuole fusion cannot be ruled out. These data only suggest that cAMP may be involved in the inhibitory action of IAA. Conclusive evidence is presented that the compound occurs; that, relative to protein, cAMP is highest in tips; that exogenous IAA affects the levels of cAMP

and that tips are the most sensitive tissues to treatment by IAA.

Attempts to localize adenyl cyclase, the enzyme responsible for cAMP synthesis, using a substrate specific for the enzyme (adenylyl imido diphosphate, AMP-PNP) and lead nitrate, revealed, with electron microscopy, discrete areas of lead precipitation on the internal membranes of cytoplasmic vacuoles of undifferentiated cells in the tips (Fig. 8). These areas correspond to previous localizations, using ATP as a substrate, of what has been described as an "acid phosphatase" (Hall et. al., 1974; Hall and Butt, 1968). Although the only membrane bound phosphatase known to be able to utilize AMP-PNP as a substrate is adenyl cyclase, it has not been demonstrated that all other phosphatases are unable to utilize it. Neither has it been demonstrated that the enzyme characterized as "acid phosphatase" is not, in fact, an adenyl cyclase. The specificity of the enzyme localized and termed phosphatase has not been determined (Sexton et. al., 1971).

In the differentiated cells of the root cap, discrete lead deposits occurred bound to the smooth endoplasmic reticulum. In light of the known secretory activity of these cells, the question arises as to whether cAMP is being secreted and transported and thus acting as a hormone, as has been demonstrated for slime molds. The data demonstrating

increases in cAMP upon incubation of tissues in IAA would not support this concept unless IAA is stimulating adenyl cyclase or inhibiting a cAMP phosphodiesterase. The decreases in total cAMP observed in tissues incubated in water (Table 2) indicate that cAMP may be leached or transported out of the tissue. Becker (1973) has reported the presence of cAMP in translocation tissues of plants. Assay of incubation media for cAMP over a time interval would quickly reveal whether this is the case. Another obvious extension of this work would be to determine whether, in fact, IAA inhibits adenyl cyclase or phosphodiesterase and in what manner. Both enzymes are readily available.

Values of cAMP determined in this study for pea hypocotyls are approximately ten times lower than values reported by Raymond (1973) for tobacco, carrot, Acer and lettuce. Values of cAMP in terms of protein in animal cells commonly fall between 50 and 100 picomoles for unstimulated tissue (Steiner et. al., 1972). At best, values determined for hypocotyls achieved half those levels. Stimulation of adenyl cyclase in animal tissue often leads to 100-fold increases in cAMP. In pea hypocotyls, a tenfold increase was observed in the most sensitive tissue. In general, the levels of cAMP are very low in the plant tissue studied and the response of tissues to hormone, in terms of cAMP production, was sluggish compared to the response of animal tissue.

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