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The Presence and Metabolism of Adenosine 3',5'-Cyclic Monophosphate in Loblolly Pine *(Pinus taeda)* Callus

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THE PRESENCE AND METABOLISM OF ADENOSINE 3',5'-CYCLIC MONOPHOSPHATE IN LOBLOLLY PINE (PINUS TAEDA) CALLUS

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SUMMARY

Adenosine 3',5'-cyclic monophosphate was found to be present in unorganized loblolly pine (*Pinus taeda*) callus grown in the dark on Winton's agar Medium 10. Cyclic AMP was assayed in callus extracts using the Gilman cyclic AMP binding protein assay under the conditions suggested by the Amersham/Searle Corporation. During a six-week growth interval following subculture, the cyclic AMP concentration in the callus varied from 24 to 345 pmoles per gram wet weight with maxima at 2 to 3 and at 5 weeks following subculture. The amounts of cyclic AMP in the callus were highest during periods of increasing callus growth rate.

The activities of both adenyl cyclase and the enzymes responsible for cyclic AMP degradation were detected in the callus. In loblolly pine callus homogenates, ATP quantities were not sufficient to saturate the adenyl cyclase and the activities of cyclic AMP degrading enzymes dominated. Enzymes catalyzing cyclic AMP hydrolysis were concentrated in the 12,100 × g pellet and were strongly inhibited by 30 mM sodium fluoride. The presence of 5'-nucleotidase activity was also detected in this fraction of callus homogenates. Enzymatic activity catalyzing adenine cleavage from both 5'-AMP and adenosine was also present; however, this activity was much lower than the activity of those enzymes catalyzing the hydrolysis of cyclic AMP to adenosine. Adenosine was the most stable product resulting from cyclic AMP hydrolysis by the callus.

Cyclic AMP was taken into the callus cell before its degradation began and the uptake of cyclic AMP by the callus was limited in the presence of 2.3 μM 2,4-dichlorophenoxyacetic acid. The 2,4-D, however, did not influence the rate of cyclic AMP hydrolysis. Tritiated activity centers in the callus resulting from incubation of the callus with [8-^{3}H]-cyclic AMP were concentrated in the cell nuclei. The presence of 2.3 μM 2,4-D did not influence the distribution of the tritiated activity centers between the nucleus and the cytoplasm.

[8-^{3}H]-Adenine derivatives were bound to some component of the 12,100 × g callus pellet fraction. 2,4-D played a role in this binding; however, it appears that the control of the binding is complex and may also be influenced by other substances naturally present in the callus.

This work was the first study of either the presence or the metabolism of cyclic AMP in tissue or cultured cells from a coniferous tree species. Although numerous published reports of the presence of cyclic AMP in higher plants have been justifiably criticized for the methods used to validate the assay response, in this work the assay response was investigated extensively and the results consistently indicated a valid assay for cyclic AMP. The finding that the endogenous cyclic AMP concentrations in the aseptic callus cultures fluctuated in a direct relationship with the growth rate is the first report of changes in cyclic AMP levels in higher plants during an extended growth period. Others have reported exogenously supplied cyclic AMP to stimulate the cellular growth rate in the presence of auxin. Utilizing a valid assay method for cyclic AMP, adenyl cyclase activity was detected for the first time in an unorganized aggregation of cells from a higher plant. Since adenyl cyclase activity has now been verified in the unorganized callus, it appears feasible that cyclic AMP could affect cellular differentiation and the tissue organization process in loblolly pine.

INTRODUCTION

Wood fiber, the basic natural resource of the pulp and paper industry, is an end product of life processes in trees. Gains have been made in increased wood fiber production as well as disease resistance through the cloning of "superior trees." In multiplying "superior tree" numbers to economically significant levels, asexual reproductive methods have the advantage over sexual reproduction in that all progeny have the same genetic composition as the parent. Therefore, asexual reproduction is the preferred method of propagating superior trees for breeding stock provided that it can be accomplished on a continuing basis with the species of interest. Gymnosperms are an important fiber source for pulp and paper; however, they are more difficult to reproduce asexually than many angiosperms. In order to realize an economically significant rate of asexual reproduction in gymnosperms, it appears that it will be necessary to gain a better understanding and control of their life processes leading to embryogenesis. Such a study is currently in progress at The Institute of Paper Chemistry using Douglas-fir (Pseudotsuga menziesii) and loblolly pine (Pinus taeda) callus cultures.

Chemical reactions in living systems occur at temperatures much lower than required for the same reactions without the catalysts of the life processes. These catalysts in living organisms are proteins known as enzymes. Hormones are substances synthesized by organisms in minute amounts, i.e., at submicromolar levels, and translocated from their site of synthesis to another part of the organism where they exert a marked influence on metabolism. This hormonal influence on metabolism is manifested either directly or indirectly through induced changes in the action of enzymes. These changes may be in the rate of enzyme synthesis, in enzyme catalytic efficiency, and/or in the substrate levels available to specific enzymes.

The cyclic nucleotides, adenosine 3',5'-cyclic monophosphate (cyclic AMP), has been shown to occupy the role of "second messenger" in the action of a number of hormones in animals and microorganisms (3). In this role, cyclic AMP levels in the hormone "target tissue" rise as a result of the hormonal influence and the increased cyclic AMP level is responsible for metabolic changes in the tissue.

It is reasonable to suspect that cyclic AMP or similar compounds might play a role in the action of plant hormones since this cyclic nucleotide is widely distributed in living organisms. Recent reports of the presence of cyclic AMP in higher plants, including a few deciduous trees, have been criticized for lack of conclusive proof of its existence. If cyclic AMP plays a role in the control of plant metabolism, it must not only be present in the plant in question, but enzymes capable of controlling its concentration levels must also be present.

HISTORICAL REVIEW

Cyclic AMP and its metabolic influences were first discovered and were studied in far greater detail in animals and microorganisms than in plants. The study of cyclic AMP in higher plants began in the last five years and only a very few of these studies have involved tree species. In this section, cyclic AMP metabolism, its effects, and known mechanisms of its action in animals and microorganisms will be reviewed briefly because almost all that is known about cyclic AMP has been determined with these organisms. The final review section contains studies of cyclic AMP and its action in plants.

CYCLIC AMP IN ANIMALS AND MICROORGANISMS

The results of cyclic AMP action in life processes have been, and likely will continue to be, observed for some time prior to its identification as the causative agent. Two examples from the past are a) the "heat stable factor" in liver homogenates which stimulated liver phosphorylase (1) and b) the chemotactic substance once called "acrasin" (2) secreted by the cellular slime mold Dictyostelium discoideum. Cyclic AMP is present in living organisms at extremely low concentrations, 0.1 to 1.0 micromoles per kg tissue wet weight in organisms other than plants (3), which accounts for the delay in the discovery of its key role as a metabolic regulator.

A number of reviews of cyclic AMP and methods used in cyclic nucleotide research are available. The book, Cyclic AMP, written by leading workers in cyclic nucleotide research (3), gives an excellent account of the determination of cyclic AMP structure and chemistry, its role in hormone action, its metabolism and processes it is known to control. The book is limited to studies of cyclic nucleotides and their effects in animals and microorganisms because at the time of its publication (1971) cyclic AMP study in plants was just beginning. Another 1971 cyclic AMP review by Jost and Rickenberg (4) covers more briefly much of the same material as Robison, et al. (3). Raven Press published the series, Advances in Cyclic Nucleotide Research in 1972 (5,6). Volume I covers the same subjects as the two previously mentioned reviews with greater emphasis on systems regulated by cyclic AMP. Volume II of the series presents assay methods for cyclic nucleotides and the enzymes controlling their levels. Chasin (7) has edited a similar volume which is a compilation of reports by various investigators. Information on some functions of guanosine 3',5'-cyclic monophosphate (cyclic GMP) is currently available but not included in the above

reviews. Cyclic GMP is currently the only 3',5'-cyclic nucleotide other than cyclic AMP known to occur naturally in living organisms (3,6,8).

Cyclic AMP Metabolism

The enzyme adenyl cyclase catalyzes the formation of cyclic AMP and pyrophosphoric acid from adenosine 5'-triphosphate (ATP) and requires either Mg^{+2} or Mn^{+2}. This enzyme has been reported in numerous tissues of animals as well as in bacteria and higher plants (4). More recently, adenyl cyclase activity has been reported in the fungus (Neurospora crassa) (9), the yeast (Saccharomyces fragilis) (10) and has been isolated in crystalline form from the bacterium (Brevibacterium liquefaciens) (11). This enzyme is associated with a cellular membrane in all organisms where it is found. The specificity of hormonal binding sites for hormones capable of stimulating adenyl cyclase activity is variable depending on the tissue or organ source (3). Liver adenyl cyclase is activated by both glucagon and epinephrine and these hormones have separate binding sites (12). The Michaelis constant (Km) of adenyl cyclase for ATP has been reported as from 0.1 to 0.5 mM in a variety of preparations (13-15). As pointed out by Robison, et al. (3), this is about an order of magnitude less than the concentration of ATP present in animal cells, but may or may not be less than the ATP concentration in contact with adenyl cyclase. Figure 1 shows the enzymes of cyclic AMP metabolism.

An enzyme capable of destroying the biological activity of cyclic AMP was found in heart, liver, and brain extracts by Sutherland and Rall (16). The product of this enzyme reaction with cyclic AMP was adenosine 5' monophosphate (5'-AMP). The requirement of Mg^{+2} as a cofactor for the cyclic nucleotide phosphodiesterase (cyclic NPDE) and the inhibition of this enzyme by caffeine were also reported. Later, it was determined that a number of methylxanthines are

effective inhibitors of cyclic-NPDE preparations from animal tissues (17). More recently, cyclic-NPDE activity has been demonstrated from a number of sources including bacteria (3), slime mold (18-20), and fungi (21). The early reviews (3-5) report that the cyclic-NPDE preparations are specific for purine 3',5'-cyclic nucleotides. Russell, et al. (22) have recently found phosphodiesterases in rat liver which have different affinities for cyclic AMP and cyclic GMP. This finding demonstrates that in the rat liver, the concentrations of these two cyclic purine nucleotides are under separate control and may play independent roles in metabolic regulation. Enzymes capable of regulating cyclic AMP levels have been found in a wide variety of organisms. These findings suggest a widespread occurrence of cyclic AMP in living systems as well as the possibility of its playing a key role in regulating metabolic processes of a wide variety of organisms.

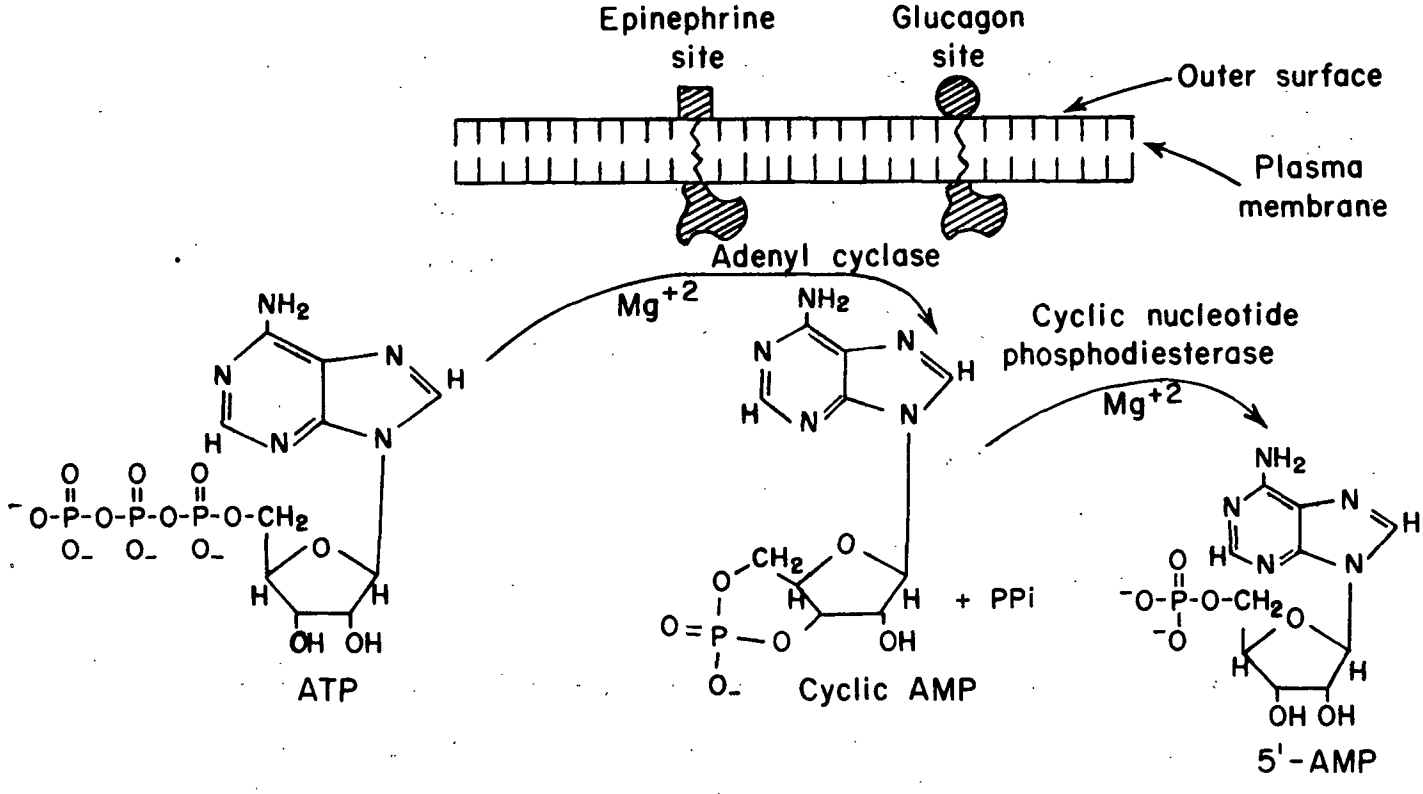


Figure 1. Cyclic AMP Metabolism in the Liver
Adapted from Robison, et al. (3)

Metabolic Control by Cyclic AMP

Cyclic AMP has been found to mediate a large number of responses attributed to hormonal control in animals, many involving the secretion of metabolites from specific organs or tissues (3). Some of these responses have been shown to result from cyclic AMP acting as a positive allosteric effector of a protein kinase. A cyclic GMP-dependent protein kinase, which phosphorylates histone, has been isolated from lobster muscle (23,24). Another protein kinase from skeletal muscle has been found to bind both cyclic GMP and cyclic AMP, but its affinities for the two cyclic nucleotides are pH dependent (8). The proteins phosphorylated by the cyclic AMP-activated kinase are usually enzymes which exist in two forms, one of which is inactive, and phosphorylation is the mechanism by which the enzyme is converted from one form to the other. Figure 2 shows how cyclic AMP controls glycogenolysis in the liver (3).

Greengard and his colleagues (25) have suggested that all of the effects of cyclic AMP may result from increased phosphorylation of a protein resulting from cyclic AMP stimulation of a protein kinase. Robison, et al. (3) have pointed out that while no evidence is inconsistent with this hypothesis and all known cyclic AMP effects require the presence of ATP, the theory certainly has not been tested for all cyclic AMP responses. In fact, the primary effect of cyclic AMP on membrane permeability could occur without the involvement of a protein. The mechanism(s) of cyclic AMP action is currently an active area of cyclic nucleotide research.

Cyclic AMP not only influences the activity of existing enzymes but also controls the synthesis of specific enzymes. In the bacterium Escherichia coli the cyclic AMP level is controlled by glucose and, at low glucose concentrations, cyclic AMP levels rise. The increased cyclic AMP induces the synthesis of

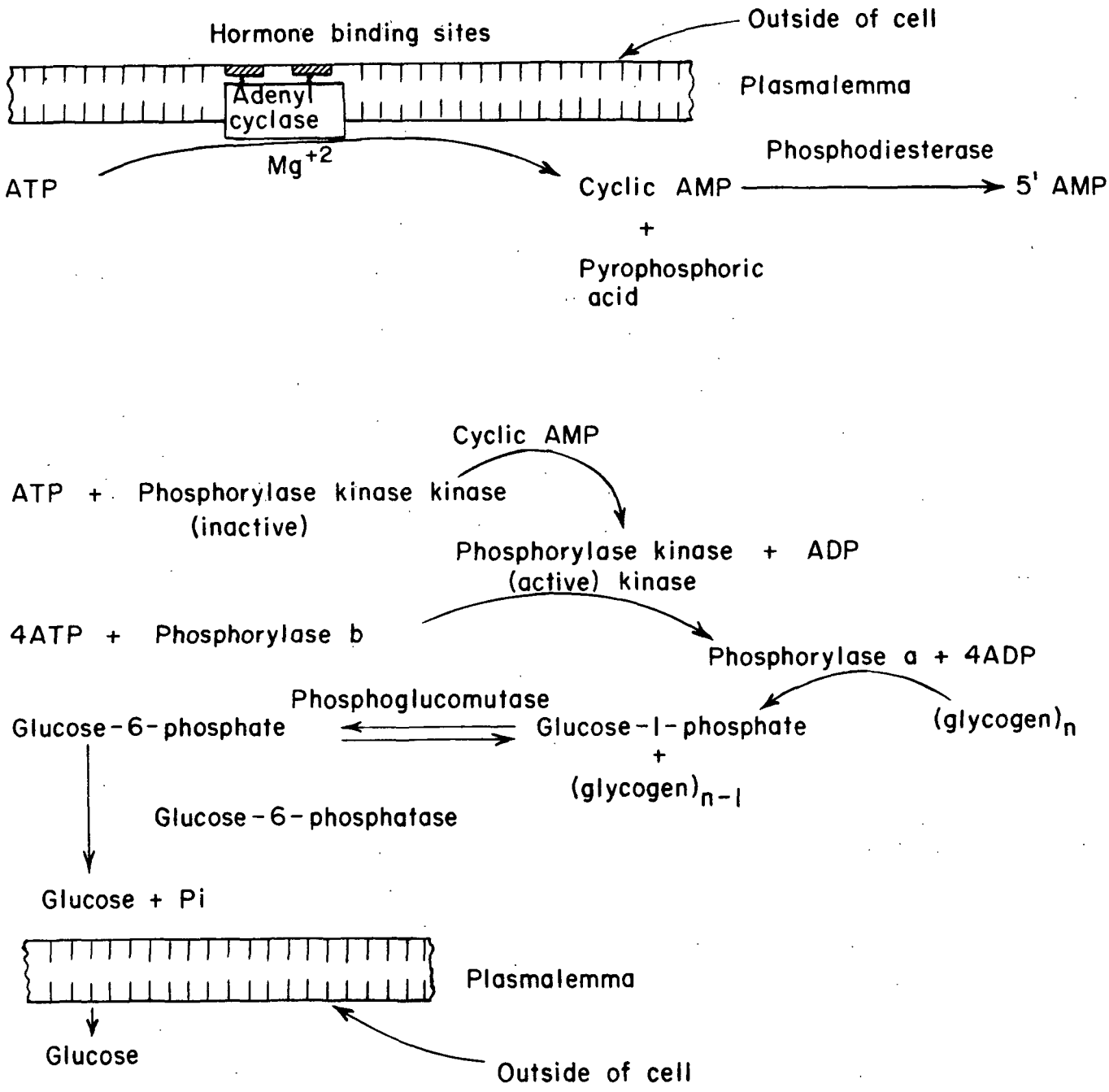


Figure 2. The Influence of Cyclic AMP on Glycogen Metabolism in the Liver. Adapted from Jost and Rickenberg (4)

enzymes capable of utilizing carbon sources other than glucose. Cyclic AMP has been shown in this case to act as an inducer of messenger RNA (mRNA) transcription from the lac operon portion of E. coli DNA. This operon is responsible for the synthesis of enzymes capable of utilizing lactose (26-28). Crombrugghe, et al. (29) found that this glucose-cyclic AMP response in bacteria is not limited to E. coli. Sy and Richter (10,30) have observed a glucose effect on cyclic AMP levels in yeast and have suggested that cyclic AMP plays a similar role in the control of enzyme synthesis as reported in bacteria. Pastan and Perlman (31) found that cyclic AMP regulates the synthesis of the enzyme tryptophanase, in E. coli at the mRNA translation level, although the mechanism is poorly understood. E. coli mutants deficient in cyclic AMP were incapable of producing indole from tryptophan without exogenous treatments with cyclic AMP.

These studies have shown how cyclic AMP plays a key role in the control of metabolic processes in many living organisms. Either hormones or glucose regulate cyclic AMP levels. Intracellular metabolic responses to these stimuli are mediated through cyclic AMP and its regulation of enzymes involved in key steps of biochemical reaction sequences.

CYCLIC AMP IN HIGHER PLANTS

The study of plant hormones and their role in the growth and development of plants has been in progress for quite some time. A number of naturally occurring plant hormones have been identified as well as some of the physiological responses of various plants to different hormones. The greatest void in the understanding of these hormones and their action lies in knowledge of the mechanism of their action. In view of the widespread importance of cyclic AMP in the action of animal hormones and its regulatory role in microorganisms, the study of cyclic AMP in plants has begun.

The investigation of the presence and physiological importance of cyclic AMP in higher plants has been in progress during the last five years. This work falls into three general categories: a) measuring and verifying cyclic AMP concentrations; b) isolating and characterizing enzymes capable of controlling cyclic AMP concentrations; and c) investigating possible roles for cyclic AMP in hormone-related responses.

Cyclic AMP Levels in Plant Tissues

Cyclic AMP levels reported in higher plants are low compared to those found in animals and microorganisms (see Table I). The methods used to extract, purify, and assay cyclic AMP in other organisms have been applied also to higher plants. A number of the reports of cyclic AMP in higher plants have been criticized recently either for a lack of definitive controls or for an incomplete reporting of experimental conditions (32). Amrhein (32) concluded that no unambiguous report of the presence of cyclic AMP in higher plants has yet been recorded. Having read an unpublished critique of cyclic AMP studies in plants by Lin (33), Amrhein reported Lin to have reached the same conclusions.

Amrhein (32) and, presumably, Lin (33) have pointed out that the radioimmunoassay of Steiner, et al. (42) and the binding protein assay by Gilman (43) both measure the displacement from a protein of a radioactive ligand ([^{3}H]-cyclic AMP or its [I^{125}]-iodotyrosine derivative) by cyclic AMP in the tissue extract. Any factor which inhibits or competes for cyclic AMP binding sites on the protein will be recorded as cyclic AMP. Therefore, further evidence is necessary to establish that the compound competing for [^{3}H]-cyclic AMP binding sites is indeed cyclic AMP.

Plant tissue investigators have used various chromatographic methods to purify their extracts. In addition, tissue extracts have been treated with

TABLE I

CYCLIC AMP LEVELS MEASURED IN PLANT TISSUES

Plant Source	Cyclic AMP Level	Assay Method	Reference
Kidney bean seedlings (6-days old)	2.6-9.2 pmole/mg dry weight	UV absorption	(34)
Kidney bean seedlings (13-days old)	0.3-0.9 pmole/mg dry weight	UV absorption	(34)
Maize seedlings	0.5-4 pmole/mg dry weight	binding protein	(35)
Honey locust sieve tube sap	9.0 μM	binding protein	(36)
Exudate from Laminaria saccharina	1.0 μM	binding protein	(36)
Oat coleoptiles	200 pmole/g fresh weight	bioluminescence	(37)
Soybean callus	11 pmole/g fresh weight	binding protein	(38)
Tobacco pith cultured tissue	84 pmole/g fresh weight	protein kinase activation	(39)
Carrot pith cultured tissue	133 pmole/g fresh weight	protein kinase activation	(39)
Sycamore suspension culture	123 pmole/g fresh weight	protein kinase activation	(39)
Chinese cabbage leaves	890 pmole/g fresh weight	bioluminescence	(39)
Lettuce seeds	269 pmole/g fresh weight	protein kinase activation	(39)
Oat coleoptiles	61 pmole/g fresh weight	radioimmunoassay	(40)
Oat coleoptiles (with 10 μM IAA)	520 pmole/g fresh weight	radioimmunoassay	(40)
Oat etioplasts (dark)	80 pmole/mg protein	binding protein	(41)
Oat etioplasts (light, 2 hr)	300 pmole/mg protein	binding protein	(41)

cyclic nucleotide phosphodiesterase as a control. Amrhein (32) pointed out that contaminating enzymes have been reported in cyclic nucleotide phosphodiesterase preparations (44) and these controls require kinetic data both in the presence of and without known cyclic AMP added to the extract. Unfortunately, those workers who have used the cyclic nucleotide phosphodiesterase treatments have not reported their reaction conditions in detail, including the specificity of the cyclic nucleotide phosphodiesterase for adenosine 3',5'-cyclic monophosphate.

Niles and Mount (45) used the Gilman assay which registered 4.0 pmoles cyclic AMP per aliquot assayed in normal broad bean (Vicia faba) tissue and 2.5 pmoles per aliquot assayed in tumor tissue. When these samples were treated with a cyclic NPDE isolated from carrot (46), the assay response went to zero. Similar extracts treated with a beef heart phosphodiesterase specific for 3',5'-cyclic AMP failed to reduce the assay response. Since the carrot cyclic-NPDE preparation was known to attack adenosine 2',3'-cyclic monophosphate as well as 3',5'-cyclic AMP, they concluded that the assay response resulted from 2',3'-cyclic AMP. This result emphasizes the difficulties which may arise from contaminating enzymes in cyclic 3',5'-nucleotide phosphodiesterase preparations without proper controls.

Amrhein (32) has used the protein kinase activation assay (47,48) to investigate cyclic AMP in various plant materials. This method measures phosphorous-32 transferred by the cyclic AMP activated kinase from γ-[^{32}P]-ATP to a protein substrate (histone). The assay has the advantage that substances other than cyclic AMP which compete for the cyclic AMP binding sites on the protein kinase will inhibit the activation of the kinase by cyclic AMP. Extracts assayed in the presence of authentic cyclic AMP should give additive kinase activation if the extract contains cyclic AMP.

Amrhein (32) investigated Robinia pseudoacacia sieve sap, Nicotiana tabacum cultured tissue, Vinca rosea (Catharanthus roseus) cultured tissue, Lycopersicon esculentum seedlings, Nicotiana tabacum pith parenchyma, and Avena sativa coleoptile sections. The Robinia pseudoacacia sap was highly inhibitory to cyclic AMP activation of the protein kinase. He reported cyclic AMP in tobacco pith parenchyma at 0.9 pmole per gram fresh weight. Apparent cyclic AMP levels in other tissues investigated were reported as upper limits for possible cyclic AMP and varied between 0.3 and 25 pmoles per gram fresh weight. These cyclic AMP levels were 1/50 to 1/1000 the levels he measured in rabbit liver by the same method. Amrhein has interpreted his data as casting doubt on the presence of cyclic AMP in higher plants; however, it seems that the data confirm the presence of cyclic AMP at very low levels. Raymond, et al. (39) (see Table I) used essentially the same methods and found cyclic AMP levels about two orders of magnitude greater than reported by Amrhein. Their evidence for the presence of cyclic AMP was based on the same criteria used by Amrhein. However, they failed to present information concerning reaction conditions in phosphodiesterase treatments used to degrade cyclic AMP and eliminate the assay responses in plant tissue extracts. Additionally, Raymond, et al. (39) have pointed out that the ratio of ATP:cyclic AMP found in germinating lettuce seeds is of the same order of magnitude as reported in animal cells.

Enzymes Capable of Controlling Cyclic AMP Levels in Plants

If cyclic AMP plays a regulatory role in higher plants, there must be an enzyme system capable of controlling its concentration. Numerous workers have investigated the activity of such a system in a wide variety of plants. All investigators to date have based their efforts upon the hypothesis that cyclic AMP synthesis and its degradation in higher plants occur by the same

reactions as reported in animals and microorganisms, i.e., adenyl cyclase and cyclic nucleotide phosphodiesterase (see Fig. 1).

Adenyl Cyclase

The two methods used most often to measure adenyl cyclase activity in higher plants are: a) the conversion of α-[^{32}P]- or [8-^{14}C]-ATP to radioactively labeled cyclic AMP and b) the conversion of [^{14}C]-adenine or [^{14}C]-adenosine to [^{14}C]-cyclic AMP. In either case, the validity of the assay is contingent upon a positive identification of the radioactively labeled product of the reaction as cyclic AMP. The problem in identification results from extremely low levels of the radioactively labeled product available for analysis.

Adenyl cyclase activity has been reported in barley aleurone layers (49), oat coleoptiles (50), maise coleoptiles (51,52), Vinca rosea tumor tissue (53), Jerusalem artichoke tubers (54), and Bengal gram seeds (55). Recently, Amrhein (32) has reinvestigated the reports of [8-^{14}C]-cyclic AMP synthesis from [8-^{14}C]-adenine by Pollard (49), Solomon and Mascarenhas (50), Janistyne (51), and Azhar and Krishna Murti (55). He found no carbon-14 activity peaks which coeluted with internal [^{3}H]-cyclic AMP standards from DEAE-Sephadex. It appears that Amrhein has a chromatographic analysis system which is superior to those used by these workers to separate possible [^{14}C]-cyclic AMP from other carbon-14 products of the reactions. His findings cast serious doubt on the earlier reports of [^{14}C]-cyclic AMP synthesis from [^{14}C]-adenine or [^{14}C]-adenosine in higher plants.

The synthesis of radioactively labeled cyclic AMP from ATP has been reported by several workers. Janistyne (52) used three chromatography systems to purify [^{14}C]-cyclic AMP to constant specific activity followed by hydrolyzing

it with barium hydroxide and identifying the products as 3'-AMP and 5'-AMP. He found that the adenyl cyclase activity was stimulated by 10 μM 3-indoleacetic acid (IAA). Wood, et al. (53) and Giannottasio, et al. (54) used only one chromatography system to characterize their radioactive-reaction products from [8-^{14}C]-ATP. Wood, et al. (53) did not test the action of plant hormones with their adenyl cyclase assay. Giannottasio, et al. (54) found that the adenyl cyclase in Jerusalem artichoke tuber homogenates was not influenced by 3-indoleacetic acid, but was stimulated by 10 μM gibberellic acid (GA_3). The validity of these reports of adenyl cyclase activity is not accepted by some investigators (32,33,46) because of the methods used to detect [^{14}C]-cyclic AMP. Janistyne's [^{14}C]-cyclic AMP characterization method appears to be adequate for proof of adenyl cyclase activity in maize coleoptiles; however, methods used by the other investigators were minimal. The presence of adenyl cyclase has been sought but not found in barley seeds (58) and cultured broad bean tissue (46).

Cyclic Nucleotide Phosphodiesterase

Phosphodiesterases capable of catalyzing the hydrolysis of 3',5'-cyclic AMP to 5'-AMP and 3'-AMP have been isolated and investigated from a number of plant sources including soybean callus (38), Jerusalem artichoke tubers (54), potato tubers (57), barley seeds (56), pea seedlings (58), Vinca rosea crown gall and normal tissue (53), and carrots (46). Some of these preparations have been called 3',5'-cyclic AMP phosphodiesterase; however, in all cases where the enzyme(s) have been purified, 2',3'-cyclic AMP is hydrolyzed as well. Nucleotide monoesterase activity has been associated with all crude cyclic NPDE preparations from plant sources reported.

Lin and Varner (58) have reported the cyclic NPDE activity in pea seedlings to hydrolyze a large number of cyclic nucleotides including both cyclic 2',3'-AMP

and cyclic 3',5'-AMP. The enzyme had a molecular weight of 350,000 and was separated from RNAase and 3'-nucleotidease activities. The 3'-nucleotidease did not catalyze the hydrolysis of cyclic 2',3'-AMP or cyclic 3',5'-AMP. Vandepeute, et al. (56) claim to have demonstrated that each of the two cyclic nucleotides competitively inhibits the hydrolysis of the other. A weakness in their work is that they were measuring inorganic phosphorus produced in the reaction which could have resulted from the hydrolysis of either the "substrate" or the "inhibitor." A number of workers have reported inorganic phosphorus to strongly inhibit plant phosphodiesterases (38,54,57,58). The inhibition observed may therefore result from the inorganic phosphorus instead of the cyclic nucleotide. Vandepeute, et al. (56) also reported that sodium fluoride inhibited cyclic 3',5'-AMP hydrolysis with little or no effects on the hydrolysis of cyclic 2',3'-AMP which suggests that the same catalytic enzyme site is not interacting with both compounds.

Cyclic nucleotide phosphodiesterase (cyclic NPDE) is present in plants in both the soluble form and bound to membranes or the cell wall. Brewin and Northcote (38) reported that whole soybean seeds were capable of hydrolyzing cyclic AMP to adenosine and whole callus cells hydrolyzed cyclic AMP without a lag period which suggested that, in soybeans, the cyclic NPDE may be located on the outside of the cell wall. Other workers have found that cyclic AMP degradation takes place following its penetration into the cell (59,60).

Cyclic NPDE from plant sources differs to some extent from the cyclic AMP degrading enzymes in animals. Methyl xanthines strongly inhibit cyclic AMP degradation in animals, but they have a small effect if any on cyclic NPDE from plants (38,53,54,56). Imidazole stimulated cyclic AMP degradation in animals, but had no effect on the cyclic NPDE from Jerusalem artichoke (54). The

isolation from Vinca rosea of a nucleotide, cytokinesin I, which was a strong inhibitor of both plant and animal phosphodiesterases, was reported by Wood, et al. (53). These results indicate that plant and animal cyclic NPDE have different regulatory sites.

Studies have usually failed to show any effect of plant hormones on cyclic NPDE activity. However, GA_3 was reported to enhance cyclic NPDE release from barley seeds and this action was inhibited by abscisic acid (56).

Cyclic AMP Effects on Metabolism and Physiology

Numerous investigations have been made of possible cyclic AMP mediation in the action of various plant hormones. Sutherland's concept of cyclic AMP as a "hormone second messenger" in animals (3) requires that the hormone be capable of stimulating an increase in the cyclic AMP level within a specific tissue and that cyclic AMP must mimic the hormone's influence on the tissue.

Reports of plant hormones stimulating the synthesis of [^{14}C]-cyclic AMP from [^{14}C]-adenine and the weakness of these reports were pointed out in the section on adenyl cyclase of plants. Using this same method, Janistyne and Drumm (61) reported rapid increases of cyclic AMP in mustard seedlings resulting from illumination with far-red light. Pollard (49) was the most diligent of the workers using this method with respect to the characterization of the radioactively labeled reaction products. He used ten chromatography systems in addition to preparing derivatives of the reaction products and all results were consistent with synthesis of [^{14}C]-3',5'-cyclic AMP. Workers using DEAE-Sephadex chromatography, however, have failed to find [^{14}C]-cyclic AMP produced from [^{14}C]-adenine (32) or hormonal stimulation of cyclic AMP synthesis.(62).

Solomon and Mascarenhas (40) reinvestigated the IAA stimulation of cyclic AMP synthesis in Avena coleoptiles using Steiner's radioimmunioassay for cyclic AMP (42). They found rapid five- to tenfold increases in cyclic AMP resulting from 10 μM IAA treatments and the cyclic AMP declined to basal levels on removing IAA. These workers depended on the specificity of the assay for cyclic AMP characterization. Wellburn, et al. (41) reported a fourfold cyclic AMP increase on exposure of oat etioplast preparations to light for two hours. They used the cyclic AMP binding protein preparation reported by Brown, et al. (63) to bind tritiated cyclic AMP synthesized from [8-^{3}H]-adenine. When the tritiated material was displaced from the binding protein and examined chromatographically, it appeared to be tritiated cyclic 3',5'-AMP. Phosphodiesterase degradation of this material yielded the products found in the degradation of authentic cyclic AMP.

A number of investigations have been made of the capacity of cyclic AMP to mimic the action of plant hormones in both physiological and metabolic responses. Robison, et al. (3) have pointed out that exogenously feeding cyclic AMP or its analogues to living cells is an abnormal method of increasing intracellular cyclic AMP concentrations and would be expected to require relatively large amounts of cyclic AMP to register a physiological response. Cyclic AMP supplements at the millimolar level have been required for physiological responses in plants. Although several metabolic and physiological responses to cyclic AMP have been observed in plants, it has not been proven that cyclic AMP acts as a "second messenger" in the action of any plant hormone.

The relationship of cyclic AMP to GA_3-controlled responses has been studied in greater detail than its relationship to any other plant hormone. Cyclic AMP has been reported to induce the synthesis of ATPase (64) and acid phosphatase (65)

in barley endosperm. These responses are also initial GA_3 responses (64). In both cases cyclic AMP responses were significantly less than optimum GA_3 responses. Nickells, et al. (65) found that both cyclic AMP and ADP induced protease synthesis with ADP being the more potent inducer. Potempa and Galsky (66) have reported that both cyclic AMP and GA_3 induce isocitrate lyase synthesis in hazel seeds. The cyclic AMP response was about 90% of the GA_3 response and, although adenine, cyclic GMP, and 3'-AMP also induced isocitrate lyase synthesis, they were not so effective as either cyclic AMP or GA_3.

Kessler and Kaplan (67) reported that when either 1 mM cyclic AMP or cyclic GMP was incubated with barley endosperm, the synthesis of gibberellic acid was stimulated. By using inhibitors and monitoring gibberellin levels via α-amylase activity, they deduced that the cyclic nucleotide induction of gibberellin synthesis was mediated through DNA synthesis. This work showed that cyclic AMP did increase GA_3 levels and also increased α-amylase activity. A number of questions related to the mechanism of cyclic AMP action still remain. How could increased DNA synthesis alone lead to increased synthesis of gibberellins specifically? Why were the inhibitors of RNA synthesis more effective against GA_3 induced α-amylase activity than against the cyclic nucleotides? Kessler and Steinberg (68) reported similarities in the action of cyclic AMP and GA_3 in flower induction and proposed that cyclic AMP induced GA_3 synthesis through stimulated DNA synthesis. In the case of barley endosperm, it appears that cyclic AMP may play more than one role in its influence on α-amylase activity. One role appears to involve RNA synthesis and its effect on gibberellin synthesis. The other role appears to be related to RNA translation, possibly for α-amylase.

The action of cyclic AMP on the rate of fresh weight increase in Jerusalem artichoke tuber slices has also been studied in some detail. Dibutyryl cyclic

AMP and GA_3 both were shown to act synergistically with auxin (2,4-D) in stimulating wet weight increase of the tuber slices (69). In the absence of auxin, neither dibutyryl cyclic AMP nor GA_3 was stimulatory. Since the effects of dibutyryl cyclic AMP and GA_3 were additive in the presence of 2,4-D their mode of action appears to be different even though their action is the same. Previous work by these investigators had shown that GA_3 action involves the stimulation of RNA synthesis (70). Kamisaka, et al. (71) reached a similar conclusion on the relationship and action of cyclic AMP and GA_3 in the study of the effect of these compounds on lettuce hypocotyl elongation and cell wall properties. Ockerse and Mumford (72) were unable to demonstrate a similar action of cyclic AMP and GA_3 in enhancing IAA induced pea stem growth. The highest concentration of cyclic AMP used in this case was 0.5 mM and a higher cyclic AMP concentration may have been required to observe a response.

These studies indicate similar actions of cyclic AMP and GA_3 in the plant tissues investigated. The mode of action of cyclic AMP in these studies remains unknown; however, it appears to be different than that of GA_3. Hartung (73) has reported that specific sugars can interfere with or enhance the action of IAA, GA_3, and cyclic AMP in oat coleoptile extension. This finding points out that all necessary controls may not have been accounted for in other similar studies.

A few studies have been conducted on the possibility of cyclic AMP as second messenger in auxin action. Solomon and Mascarenhas (74) found cyclic AMP, cyclic GMP, and 3'-AMP to delay Coleus leaf petiole abscission. Auxins are well known for delaying leaf abscission when applied to the distal end of the petiole. The limited number of control compounds and the lack of specificity found leaves doubt that cyclic AMP was acting as a second messenger for IAA in this study.

These workers also reported accelerated RNA synthesis by chromatin isolated from oat coleoptiles in the presence of either IAA or cyclic AMP (75). This effect was over and above the possible influence of other nucleotides present in the incubation medium. When IAA and cyclic AMP were both present at optimal levels, their effects on RNA synthesis were neither additive nor synergistic. This observation indicates that IAA and cyclic AMP were acting in the same manner to stimulate RNA synthesis. One vital question which remains is whether or not both cyclic AMP and IAA were stimulating the synthesis of the same type of RNA.

In summary, many of the reports of the presence of 3',5'-cyclic AMP and stimulated increases in its levels in plants are not supported by sufficient controls. The experiments reported by Amrhein (34), Raymond, et al. (41), and Wellburn, et al. (43), however, do verify the presence of cyclic AMP in some higher plants. Cyclic AMP levels appear to be increased in oat etioplasts on exposure to light (43). All reports of adenyl cyclase in higher plants except those by Pollard (49) and Janistyne (52) can be questioned because of the basis for characterizing the radioactivity labeled reaction product. The cyclic NPDE in higher plants, unlike the cyclic NPDE's of animals and microorganisms, is neither activated by imidazole nor inhibited by methylxanthines. Plant cyclic NPDE's are not specific for 3',5'-cyclic nucleotides; they also catalyze the hydrolysis of 2',3'-cyclic nucleotides. Reports of hormone-induced increases in cyclic AMP levels are only as good as the verification of the presence of cyclic AMP. Except for the reports by Pollard (49) and Janistyne (52), there is reasonable doubt of hormonal influence on cyclic AMP concentration in plants. Cyclic AMP seems to have the same effect on barley endosperm and Jerusalem artichoke tubers as GA_3 does, although its mode of action is different. Cyclic AMP stimulates GA_3 synthesis in barley endosperm and hazel seeds and appears to control the synthesis of specific enzymes at both the RNA transcription and translation steps. The

mode of action of cyclic AMP in these instances is unknown. Cyclic AMP-dependent protein kinase activity has not been reported in higher plants. Cyclic AMP action in auxin-controlled responses has been investigated in only a few plant tissues and no clear relationship between cyclic AMP and auxins has been demonstrated. Cyclic AMP has not been shown to act as a second messenger in the action of any of the plant hormones investigated, however, it does appear to be a GA_3 inducer which responds to light stimulus.

THESIS OBJECTIVES AND APPROACH

The historical review points out criticism of reports that adenosine 3',5'-cyclic monophosphate and adenyl cyclase are present in plants. These critical commentaries have come during the progress of this thesis and have influenced the thesis objectives. The initially proposed objectives were to determine whether auxin treatments of undifferentiated aspen callus cells resulted in modulation of cyclic AMP concentrations and to identify system(s) on which the auxins acted if changes in cyclic AMP levels occurred.

The abovementioned commentaries on evidence for the presence of cyclic AMP and adenyl cyclase in plants pointed out weaknesses in the evidence in the literature and it became obvious that the initially proposed thesis objectives were premature. The primary objective of this thesis then became to determine whether cyclic AMP and enzymes capable of controlling its concentration are present in unorganized loblolly pine (Pinus taeda) callus cultures. The influence of auxin on cyclic AMP concentration became a secondary objective. Although some auxin influences were studied, the defined thesis objectives excluded the mechanism of auxin action. These objectives were chosen for the purpose of establishing a basis for future studies of a possible role of cyclic AMP as a metabolic regulator in loblolly pine.

A callus culture was chosen for the study because it is grown on a defined medium and under environmentally controlled conditions which are desirable for this study. Also, this undifferentiated form of tree cell is being used to study the differentiation process here at The Institute of Paper Chemistry.

METHODS AND MATERIALS

LOBLOLLY PINE CALLUS SOURCES

Loblolly pine (Pinus taeda) callus used in this study was initiated on Winton's agar Medium 10 (Appendix I), June 14, 1971. Explants from the current year's branch-growth of a tree in North Carolina were used in the initiation. Callus which formed was subcultured onto Medium 10 and grown in the dark at 27 ± 1°C with subculturing at three week intervals.

The loblolly pine callus grown on Winton's agar Medium IPA-2 (Appendix I) was from the callus initiation reported above. In September, 1973, callus was subcultured from Medium 10 to agar Medium IPA-2 and thereafter subcultured at 4 to 5 week intervals on IPA-2.

CYCLIC AMP ASSAY

CALLUS EXTRACTION AND EXTRACT PREPARATION FOR ASSAY

Extracts from callus were partially purified prior to assay of cyclic AMP with a cyclic AMP binding protein assay kit purchased from Amersham/Searle Corporation. The cyclic AMP level in the callus was stabilized by freezing the callus in liquid nitrogen. Cyclic AMP was extracted and proteins precipitated with ice-cold 6% trichloroacetic acid (TCA). TCA was removed from the extracts with water-saturated ether. The procedure is reported in detail in Appendix II and is similar to the method used by Gilman (43).

The lyophilized callus extracts were each redissolved in 1 ml of 8.3 mM tris-HCl (pH 7.5) buffer and eluted through 1 g of neutral chromatographic alumina, Grade I, Type WN-3 (Sigma Chemical Co.) contained in glass columns of 6-mm inside diameter. The columns were prepared as described by White and

Zenser (76) except that the elution buffer was 8.3 mM tris-HCl (pH 7.5) instead of 50 mM tris-HCl (pH 7.6). Appendix II, Table VII, shows the elution of a number of nucleotides, nucleosides, and nitrogenous bases from alumina reported by these workers. This chromatographic system was particularly well suited for preassay purification of extracts to be assayed with the Amersham/Searle kit because the column elution buffer is similar to the cyclic AMP assay buffer.

For each set of extracts to be chromatographed on several separate columns at the same time, the eluate from one column was collected in 1.0-ml fractions and 0.1-ml aliquots of each fraction were assayed for tritiated activity. All other extracts in that set were collected from separate alumina columns prepared at the same time based on cyclic AMP elution from the first column. Appendix II, Fig. 28, shows the elution of [8-^{3}H]-cyclic AMP in this chromatography system and the second through the fourth milliliter of eluate was collected, lyophilized, and reconstituted to 0.5 ml with 4 mM disodium ethylenedinitrilotetraacetate (EDTA). The extracts reconstituted in this manner were in the Amersham/Searle assay buffer: 50 mM tris-HCl (pH 7.5) + 4 mM EDTA. Aliquots of the reconstituted extracts were counted for tritium by liquid scintillation to estimate cyclic AMP recovery. Fifty-microliter aliquots of these solutions were assayed for cyclic AMP content.

CYCLIC AMP BINDING PROTEIN ASSAY

The cyclic AMP assay system purchased from Amersham/Searle Corporation utilizes the competition between cyclic AMP and [8-^{3}H]-cyclic AMP for binding sites on a protein kinase which has a high specificity for cyclic AMP. This protein kinase was isolated from bovine muscle by the procedure reported by Gilman (43). The assay was performed according to the procedure recommended by Amersham/Searle except that 100-µl aliquots of assay supernatant were counted

for tritiated activity rather than the recommended 200-µl aliquot. Generally, the assay involves the establishment of a binding equilibrium between the assay protein, [8-^{3}H]-cyclic AMP and unlabeled cyclic AMP which may be present in a standard solution or in a callus extract. After equilibration for 2 hours, unbound cyclic AMP was adsorbed on activated charcoal and, following charcoal sedimentation by centrifugation at 9950 × g for 8 minutes at 2°C, an aliquot of the supernatant was counted for tritiated activity. Samples and assay standards were routinely assayed in duplicate. Pipetting was done with Centaur constant volume pipets fitted with disposable plastic tips. The pipet tip was shortened 1 cm for pipetting charcoal suspensions as recommended by Amersham/Searle.

Appendix III shows a cyclic AMP assay standard curve and a sample calculation of cyclic AMP concentration.

3',5'-CYCLIC NUCLEOTIDE PHOSPHODIESTERASE REACTIONS

The 3',5'-cyclic nucleotide phosphodiesterase preparation used to investigate the cyclic AMP binding protein assay response was purchased from Sigma Chemical Company as prepared by the procedure reported by Butcher and Sutherland (17) through the DEAE-cellulose chromatography step. Enzyme activities associated with the Sigma preparation are listed in Appendix IV. The callus extracts used in phosphodiesterase reactions were first chromatographed on alumina columns. Reaction conditions for the specific reactions are reported in the Results and Discussion section.

LIQUID SCINTILLATION COUNTING

All liquid scintillation counting was done with the Beckman LS-100 liquid scintillation counter. The counting cocktail used was Beckman Cocktail D which consisted of 100-g naphthalene (AR grade, J. T. Baker Chemical Co.) and 5-g

2,5-diphenyloxazole (scintillation grade, Interex Corp.) per liter solution in 1,4-dioxane (scintillation grade, International Chemical and Nuclear Corp. or J. T. Baker Chemical Co.). Each sample was counted in 10 ml of Beckman Cocktail D.

In counting radioactive compounds in scrapings from thin-layer plates, 0.5-ml methanol was added to the counting vial containing the coating before Cocktail D was added. Scrapings from MN Polygram Cel_{300} UV_{254} coated plates which contained [^{3}H]-cyclic AMP were counted for tritiated activity within one hour of adding Cocktail D since [^{3}H]-cyclic AMP was found to rebind to the coating and the tritiated activity became quenched (see Appendix V). The counts of scrapings from these plates containing other tritium-labeled adenine derivatives were either constant or increased with time. These samples were recounted until the difference between the last two counts was within the counting error.

CALLUS GROWTH AND CYCLIC AMP CONCENTRATIONS FOR SIX WEEKS FOLLOWING SUBCULTURE

For growth measurements, 10 pieces of callus in one set were weighed and returned to the agar medium each week with dry weight determinations at the end of six weeks. Two additional sets of 10 pieces each were selected for both wet and oven-dry (42°C for 21 hours) weights at the beginning and end of the growth passage. At the end of the six-week passage, the dry weight:wet weight ratio was not significantly different between callus pieces left undisturbed for the duration of the study and those removed each week for weighing and returned to the agar.

The callus handling procedure in the growth study prior to cyclic AMP extraction was somewhat different than the routine method previously described. Here, the objective was to observe as many physical characteristics as possible of the callus assayed. The callus to be assayed was therefore photographed

prior to freezing it in liquid nitrogen. Callus exposure to photographic lighting did not exceed 40 seconds and a control indicated that the light received during photography did not influence cyclic AMP levels in the callus (Appendix VI).

PROTEIN ASSAYS

The microbiuret method reported by Koch and Putnam (77) was used to determine the protein content of loblolly pine callus preparations used for adenyl cyclase and cyclic AMP degradation reactions. Correction for turbidity was via their method of subtracting twice the absorbance at 392 nm from the absorbance at 330 nm. Solutions of known protein concentration were prepared for standard curve construction using bovine serum albumin (Pierce Chemical Co.).

ADENYL CYCLASE ASSAYS

FORMATION OF CYCLIC AMP FROM ATP

Adenyl cyclase activity was assayed in loblolly pine callus whole cells and in both fractions resulting from sedimenting a callus homogenate by centrifugation at 600 × g for 10 minutes at 2°C. The callus used in this investigation had grown on agar Medium 10 in the dark for 12 days since subculture. It was homogenized by hand in a ground-glass homogenizer at 2°C with 1 ml of 50 mM tris-HCl [(pH 7.5) containing 5 mM magnesium chloride] per gram callus wet weight. The 600 × g pellet was redispersed in homogenizing buffer (ml/g) equal to the initial callus wet weight by sonication with a General Electric Ultrasonic Generator. The samples were sonicated at 150 ma with cooling until the pellet was well dispersed (usually about 15 minutes). The 600 × g supernatant fraction was assayed without further treatment. Whole callus cells to be assayed were suspended in two volumes (ml/g) of the homogenizing buffer and were agitated on a

Dubnoff Metabolic Shaking Incubator at 30°C for one hour prior to use in adenyl cyclase assays.

Adenyl cyclase reaction mixtures contained the following:

100 µl callus preparation

50 µl 50 mM tris-HCl (pH 7.5) containing 15 mM magnesium chloride, 90 mM sodium fluoride, 3 mM dithiothreitol, and 6 mM disodium 5'-adenosine triphosphate (Na_2ATP)

Control reactions were run with only the Na_2ATP eliminated from one incubation and with only the sodium fluoride eliminated from the other incubation. All reactions were run in duplicate for each reaction time at 30 ± 1°C on the Dubnoff incubator and reactions were quenched by immersing reaction tubes in a boiling water bath for 3 minutes. The homogenizing and assay buffers were modified from those used by Wood, et al. (53).

After sedimenting the solids from the reaction mixtures by centrifugation, 50-µl aliquots of the supernatants were assayed in duplicate using the Amersham/Searle Corp.'s cyclic AMP binding protein assay kit. Cyclic AMP standard solutions for standard curves in this case were prepared in adenyl cyclase assay buffer. A list of compounds reported by Amersham/Searle Corp. as not interfering with the assay is presented in Appendix VII.

[8-^{14}C]-ADENINE INCUBATIONS

An attempt was made to synthesize [8-^{14}C]-cyclic AMP from [8-^{14}C]-adenine with loblolly pine callus. The methods used were those reported by other workers using tissue from various plant sources (32,49,51). In this adenyl cyclase assay, [8-^{14}C]-ATP was synthesized in vivo from [8-^{14}C]-adenine and utilized as the adenyl cyclase substrate.

Callus Incubation and Nucleotide Extraction

Twenty pieces of loblolly pine callus, grown in the dark for 30 days since subculture, were suspended in 50 ml of 50 mM potassium phosphate buffer (pH 6.25) containing 0.1 mM theophylline and 5 μCi [8-^{14}C]-adenine, specific activity 54 mCi/mmole (International Chemical and Nuclear Corp.). The incubation buffer and flask were autoclaved and cooled before adding the [8-^{14}C]-adenine and callus. Callus cells were incubated for 3 hours at 28 ± 1°C using the Dubnoff incubator. At the end of the incubation, callus cells were collected on cheesecloth and the nucleotides extracted in the same manner as previously described (Appendix II). The incubation medium was lyophilized following callus removal.

Cyclic AMP Isolation

Activated Charcoal

The lyophilized callus extract and incubation medium were both brought to 8 ml with distilled water, then 80-mg Norit SG-Extra (J. T. Baker Chemical Co.) was added to each, the suspension stirred, and the charcoal sedimented by centrifugation. Charcoal pellets were each then mixed with 4 ml 50% ethanol containing 2% ammonium hydroxide and the charcoal was sedimented by centrifugation. This treatment was repeated three times to displace nucleotides bound to the charcoal. The combined supernatants from each pellet were neutralized, lyophilized, and redissolved in 1 ml distilled water.

Bio-Gel P-2 Chromatography

The charcoal eluates (above) were first chromatographed through alumina as previously described and the fraction containing cyclic AMP was collected. The incubation medium was chromatographed on the Bio-Gel P-2 column without any pre-purification steps. Both the callus extract and the incubation medium contained added nucleosides, nucleotides, and nitrogenous bases at 10 to 20 mM concentrations

as well as [8-^{3}H]-adenosine 3',5'-cyclic monophosphate as internal standards. The transmission of the column eluate was monitored at 254 nm with a LKB Uvicord, then collected at 7-minute intervals.

Nucleotides in the callus extract and in the incubation medium were separated using a Bio-Gel P-2 (<400 mesh) column under similar conditions to the technique reported by John, et al. (78,79). The column dimensions were 1.5 cm ID × 2.75 m. The chromatography setup is illustrated and details of column packing described in Appendix VIII. Samples of 50 or 100-µl volume were injected on the column and eluted with degassed 0.25M citrate buffer (pH 4.4) at a flow rate of about 36 ml per hour. The column temperature varied from 74°C at the outflow end to 69°C at the top of the column. The elution of standards is shown in Appendix VIII (Table XII).

CYCLIC AMP DEGRADATION ASSAYS

Cyclic AMP degradation catalyzed by enzymes from loblolly pine callus was assayed using a chromatographic method similar to the one reported by Wood, et al. (53). Callus was homogenized by hand in a TenBroeck ground-glass homogenizer at 2°C with an equal amount (g/ml) of 1 mM tris-HCl (pH 7.5) buffer containing 2-mM dithiothreitol and 2-mM magnesium chloride. The resulting homogenate was fractionated by centrifugation at 2°C in one of two ways, with or without an initial centrifugation at 600 × g for 10 minutes. The remainder of the fractionation procedure included sedimentation at 12,000 × g for 20 minutes, then recentrifuging the 12,000 × g supernatant at 100,000 × g for 2 hours. Fractions assayed included the 600 × g pellet, 12,100 × g pellet,* 100,000 × g pellet, and the 100,000 × g supernatant. All pellet fractions were resuspended

*Unless otherwise indicated, solids sedimenting at 600 × g for 10 minutes were excluded in 12,100 × g pellet preparations.

in homogenizing buffer and sonicated at 150-175 ma with cooling until well dispersed using a General Electric Ultrasonic Generator.

When the degradation of cyclic AMP by a suspension of whole callus cells was studied, the callus was suspended in an equal amount of homogenizing buffer (g/ml) and agitated at 30°C in a Dubnoff incubator for 45 to 60 minutes before use in assays. Whole callus cell suspensions were pipetted with a Centaur micropipet in which about 1 cm was removed from the disposable plastic tip.

Reaction mixtures contained the following:

50 µl callus preparation as above

50 µl 50-mM tris-HCl (pH 7.5) with 5-mM magnesium chloride, 1-mM dithiothreitol, and 0.2-mM cyclic AMP

20 µl [8-^{3}H]-cyclic AMP, specific activity 21.8 Ci/mmole (30,000 cpm except as noted) purchased from International Chemical and Nuclear Corp.

Reactions were initiated by adding the callus preparation and were terminated either by heating the reaction tubes in a boiling water bath or by adding 1 ml of 6% TCA (Appendix IX). After reaction termination, 10 µl each of 0.2-mM 5'-AMP, 0.2-mM 3'-AMP, and 0.2-mM adenosine were added to each of the reaction mixtures. Reaction mixtures were then lyophilized (following TCA removal with ether if TCA-terminated) and redissolved in 0.2-ml distilled water. Solids were then sedimented by centrifugation and 100-µl aliquots of supernatant chromatographed by thin-layer chromatography (TLC). In whole cell reactions, reaction mixtures were sonicated to break cell walls before they were centrifuged.

The thin-layer chromatography system routinely used to separate reaction products was Machery-Nagel MN Polygram Cel_{300} UV_{254} coated plates developed with n-butanol-methanol-ethyl acetate-concentrated ammonium hydroxide (7:3:4:4, v/v) (49). Adenosine, 5'-AMP, 3'-AMP, cyclic AMP, and adenine were routinely used as chromatography standards and their migration was detected by UV absorption. The development time was about 3 hours. Following development and spot detection, the thin-layer coating was scraped from the plates with a razor blade for tritium counting by liquid scintillation. An example of the migration of the above compounds with this TLC system and the tritiated activity distribution is shown in Appendix X (Fig. 31).

CYCLIC AMP DEGRADATION WITH VARYING AMOUNTS OF 12,100 × g CALLUS HOMOGENATE PELLET

These reactions were run using varying amounts of the 12,100 × g pellet from loblolly pine callus grown on agar Medium IPA-2 in the dark for 25 days since subculture. Assays were carried out as previously described except that the assay buffer containing 18,000 cpm [8-^{3}H]-cyclic AMP was constant at 50 µl and the callus pellet fraction was varied from 10 to 50 µl with homogenizing buffer added as needed for total reaction volumes of 100 µl. The reaction time was 10 minutes for all reactions.

ADENOSINE MONOPHOSPHATE TRAPPING

Adenosine monophosphate intermediates occurring in [8-^{3}H]-cyclic AMP enzymatic hydrolysis to [8-^{3}H]-adenosine were determined by trapping the [8-^{3}H]-AMP with 3'-AMP and 5'-AMP. For this purpose, [8-^{3}H]-cyclic AMP degradation reactions were run as previously described with the 12,100 × g pellet fraction except the assay buffer contained 20-mM 5'-AMP and 21-mM 3'-AMP. Reaction times were 10 minutes with termination by heat. The TLC system used to

separate 3'-AMP and 5'-AMP in the reaction mixture was silica gel F-254 coated aluminum plates (Brinkmann Instrument Co.) which were developed with propanol-2-concentrated ammonium hydroxide-water (70:15:15, v/v). Chromatographic results are shown in Appendix X (Fig. 32).

5'-NUCLEOTIDASE ASSAY

5'-Nucleotidase activity was assayed in the 12,100 x g callus homogenate pellet. These reactions were run using the same homogenizing and assay buffers previously described for [8-^{3}H]-cyclic AMP degradation assays. In the 5'-nucleotidase assay, the [8-^{3}H]-cyclic AMP solution was replaced by 20 µl of homogenizing buffer containing 45,800 cpm [8-^{14}C]-5'-adenosine monophosphate, disodium salt, specific activity 57.4 mCi/mmole (International Chemical and Nuclear Corp.). Reaction termination was by TCA addition. The standard TLC separation of reaction products was used (Appendix X, Fig. 31).

[8-^{14}C]-ADENOSINE AS SUBSTRATE FOR ADENYL CLEAVING ACTIVITY

The [8-^{14}C]-adenosine for these reactions was prepared from [8-^{14}C]-5'-adenosine monophosphate disodium salt (specific activity 57.4 mCi/mmole) by hydrolysis catalyzed by the nucleotidase from the callus and purified by TLC as described for the 5'-nucleotidase assay. The [8-^{14}C]-adenosine was extracted from the coating of the thin-layer plate with hot water after scraping the coating off the plate. A glass wool microfilter was prepared in the barrel of an eye dropper for this purpose. The specific activity of the [8-^{14}C]-adenosine isolated by TLC (nonradioactive adenosine was added for detection by UV absorption) was calculated using the molar absorptivity of adenosine reported by Pabst Laboratories, Inc. (80). Absorbance measurements were made at 259 nm using a Beckman DU spectrophotometer.

The enzymatic reactions contained the following components:

50 µl — 12,100 × g callus pellet preparation

50 µl — [8-^{14}C]-adenosine in homogenizing buffer (5100 cpm)

50 µl — cyclic AMP degradation assay buffer as previously described

Reactions were run in duplicate for 0, 5, 10, and 15 minutes and were quenched by heating reaction tubes in a boiling water bath for 3 minutes. Reaction products were separated by TLC as previously described for [^{3}H]-cyclic AMP degradation reactions.

AUXIN TREATMENTS

[8-^{3}H]-Cyclic AMP degradation in the presence of auxin was investigated using the same method as previously described for studying cyclic AMP degradation except for the auxin additions. In these reactions, auxin was included as either 2.3-µM 2,4-D or 10-µM IAA. Controls with no auxin added were run with the same callus preparations. [8-^{3}H]-Cyclic AMP (27,000 cpm per reaction) was used in this study. These reactions were run on two different occasions using separate callus preparations. The first was callus which had grown 21 days since subculture and, in the repeat experiment, the callus had grown 15 days since subculture.

Treatment of the 12,100 × g solids with n-butanol-methanol-ethyl acetate-concentrated ammonium hydroxide (7:3:4:4, v/v) was by different methods for the two reaction sets. In the first set of reactions, the pellet was mixed with the supernatant following reaction termination and centrifugation and 0.3 ml of the treatment solution was added to 0.1 ml of the suspension in the reaction tube.

Aliquots of 50-µl volume were counted for tritium. In the second reaction set, 50-µl aliquots of reaction suspensions were added to liquid scintillation counting vials with or without the subsequent addition of the treatment solution.

LIGHT MICROSCOPE AUTORADIOGRAPHIC LOCALIZATION OF [8-^{3}H]-CYCLIC AMP AND PRODUCTS OF ITS DEGRADATION IN LOBLOLLY PINE CALLUS

The callus used in this study had grown on Medium 10 for 21 days after subculture. The basic incubation medium was 2-ml 50 mM tris-HCl (pH 7.5) buffer containing 0.1M D-glucose, 10-mM magnesium chloride, and 0.9-mM dithiothreitol. Four different treatments were made with the following additions to the basic incubation medium:

1. 60-µM Adenosine 3',5'-cyclic monophosphate (Nutritional Biochemicals Corp.) and 1,898,000 cpm [8-^{3}H]-cyclic AMP, specific activity 21.8 Ci/mmole.
2. The same additions as in 1; the medium also contained 2.3-µM 2,4-D.
3. 60-µM Guanosine 3',5'-cyclic monophosphate, potassium salt, grade B (Calbiochem) and 1,862,000 cpm [8-^{3}H]-guanosine 3',5'-cyclic monophosphate, specific activity 21.6 Ci/mmole.
4. The same additions as in 3; the medium also contained 2.3-µM 2,4-D.

The callus was incubated with [8-^{3}H]-cyclic AMP and [8-^{3}H]-cyclic GMP for two hours at 30 ± 1°C in a Dubnoff incubator. At the end of the incubation, the incubation medium was decanted off and the callus prefixed with a mixture of 2% glutaraldehyde and 2% acrolein in cacodylate buffer (0.05M at pH 7.0). The fixation and embedding schedule was by procedure No. 5 used in IPC Project 3223 (81). The sectioning, staining, and autoradiography technique was done with the same equipment, chemicals, and techniques reported by Mullis (82) for autoradiography as applied to light microscopy.

The finished sections were photographed, as viewed in phase contrast through a Zeiss Photomicroscope Model I, with a 35-mm camera. Ten cells of each treatment with [8-^{3}H]-cyclic AMP present were photographed. Tritiated activity centers per unit area were determined by counting developed silver grains and measuring areas with a planimeter from 3 × 5 inch prints. Two exposures were taken of each cell, one with the silver grains in focus and the other with the callus cell in focus. Silver grain counts and area determinations were made for the nuclei, cytoplasm, and vacuoles.

AUXIN EFFECT ON EXTRACTABLE CYCLIC AMP FROM CALLUS WHOLE CELLS

Loblolly pine callus which had grown on agar Medium 10 for 14 days since subculturing was treated in place by dropping solutions of 2,4-D on the callus. Treatment solutions used were double-distilled water, 2.3-μM 2,4-D, and 23-μM 2,4-D. Two drops of treatment solution were placed on each callus mass and samples for cyclic AMP assay consisted of 2 callus masses each. Covered Petri dishes containing the treated callus were wrapped in aluminum foil until treatment was terminated. Duplicate samples were prepared for each treatment at 5, 10, 20, and 60-minute treatments. The treatments were terminated by immersing callus pieces in liquid nitrogen. The cyclic AMP extraction and assay were carried out as previously described.

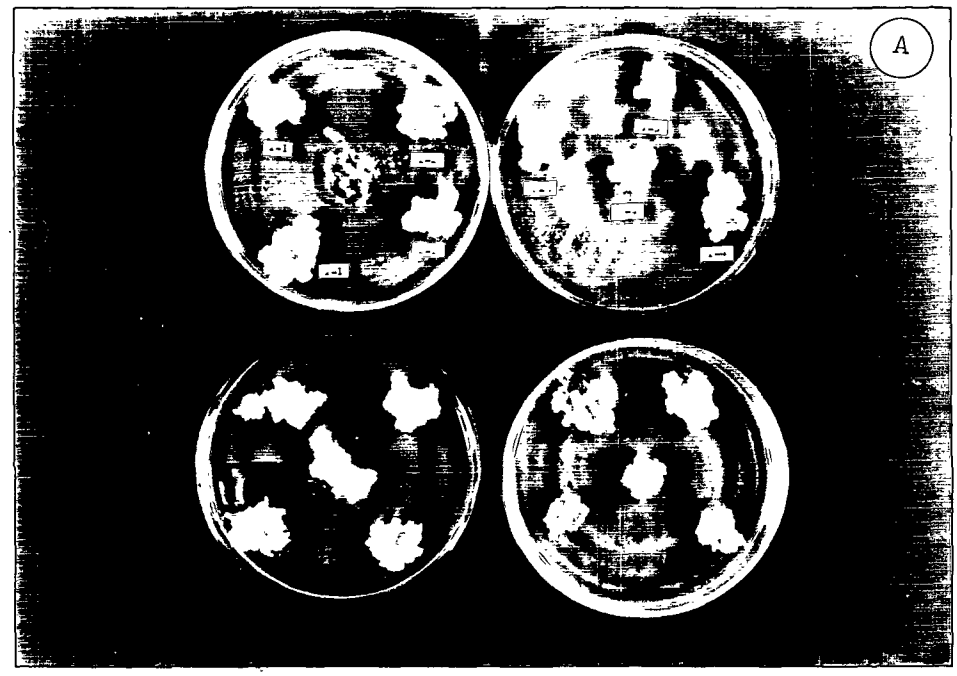
RESULTS AND DISCUSSION

In this section, loblolly pine callus growth characteristics and observed relationships between these characteristics and cyclic AMP concentration are described. Methods used to verify the cyclic AMP assay are then presented. Lastly, results of the investigation of enzymes capable of regulating cyclic AMP concentration in loblolly pine callus are presented.

LOBLOLLY PINE CALLUS MORPHOLOGY AND GROWTH CHARACTERISTICS

Loblolly pine callus grown in the dark on Winton's Medium 10 (Appendix I) was soft to mushy, becoming crumbly in texture during the fifth and sixth weeks after subculture. As shown in Fig. 3, the callus grew as an irregular cellular mass which was light yellow (Fig. 3A) at two weeks after subculture and became tan, occasionally reddish brown (Fig. 3B) by the sixth week. Callus in the two bottom Petri dishes of Fig. 3A and 3B were the same pieces photographed at two and six weeks, respectively, following subculture. These pieces were removed and weighed weekly to determine callus fresh weight increase with time. Callus in the four top Petri dishes was used for cyclic AMP assay as part of a study where the cyclic AMP level in the callus was measured at weekly intervals for six weeks following subculture. It is apparent from Fig. 3 that the tissue darkening with time is more prominent in the callus which was removed weekly for weighing than that which remained on the agar medium continuously. No evidence of organ or tissue differentiation was observed within the callus during the thesis investigation.

Loblolly pine callus cells were typically highly vacuolated with only occasional cells containing more cytoplasm than vacuole by volume. Figure 4



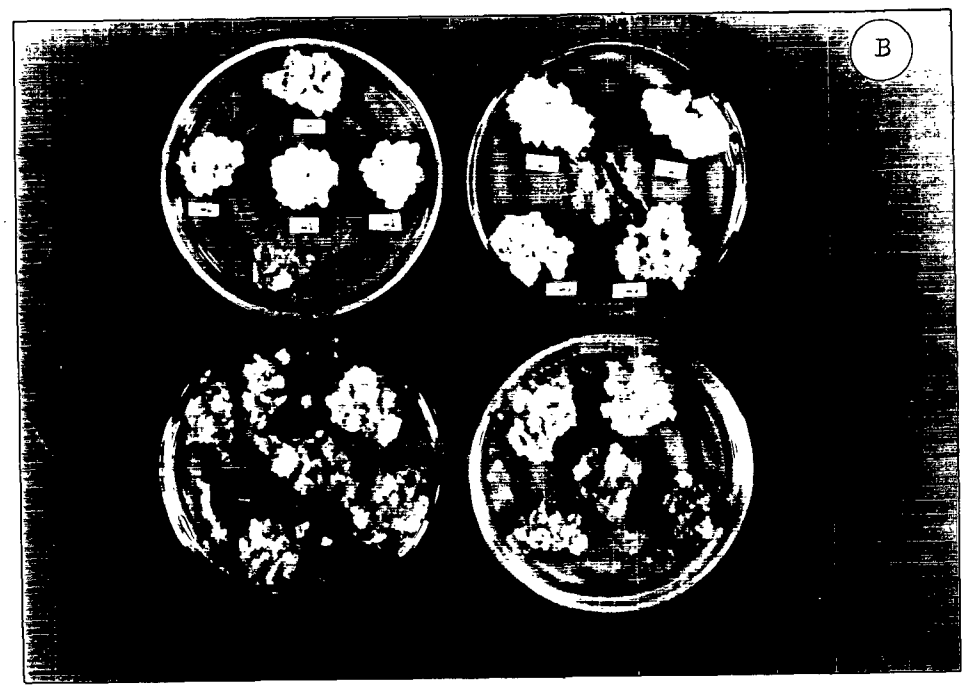


Figure 3A and 3B. Loblolly Pine Callus Grown in the Dark on Medium 10. A, 14 Days After Subculture; B, 42 Days After Subculture

shows callus cells with relatively large amounts of cytoplasm. The callus cell walls consisted of a thin primary wall about 0.5-μm thick with no apparent secondary thickening.

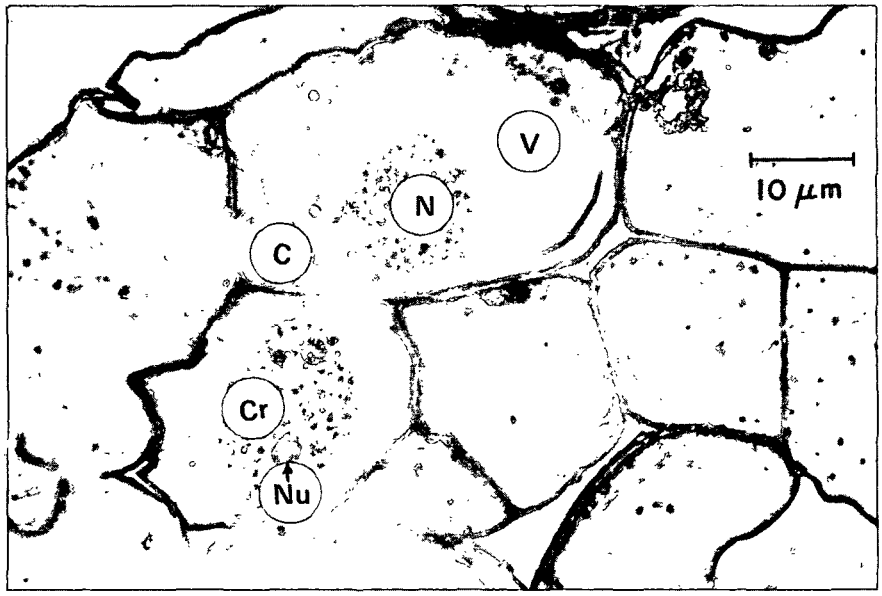


Figure 4. Section Through Loblolly Pine Callus Fixed with Glutaraldehyde and Osmium Tetroxide and Stained with 0.05% Toluidine Blue (pH 6.8). V = Vacuole; N = Nucleus; C = Cytoplasm; Nu = Nucleolus; Cr = Chromatin 1400X

Figure 5 shows cumulative callus fresh weight with time from subculture. The lag phase of growth continued through the first week followed by a log growth phase during the second and third weeks. The callus fresh weight continued to increase at a reduced rate during the fourth and fifth weeks. The decline in average fresh weight during the sixth week from subculture resulted from the weight contribution of those pieces on the Petri dish from which portions of agar were removed (see Fig. 3). It was necessary to remove the agar from these areas because of bacterial contamination. Callus growing under these conditions without agar removal may have continued to increase its fresh weight during the sixth week at a slower rate than in the preceding week as indicated by the dashed line in Fig. 5. From beginning to end of the six-week growth period, the average

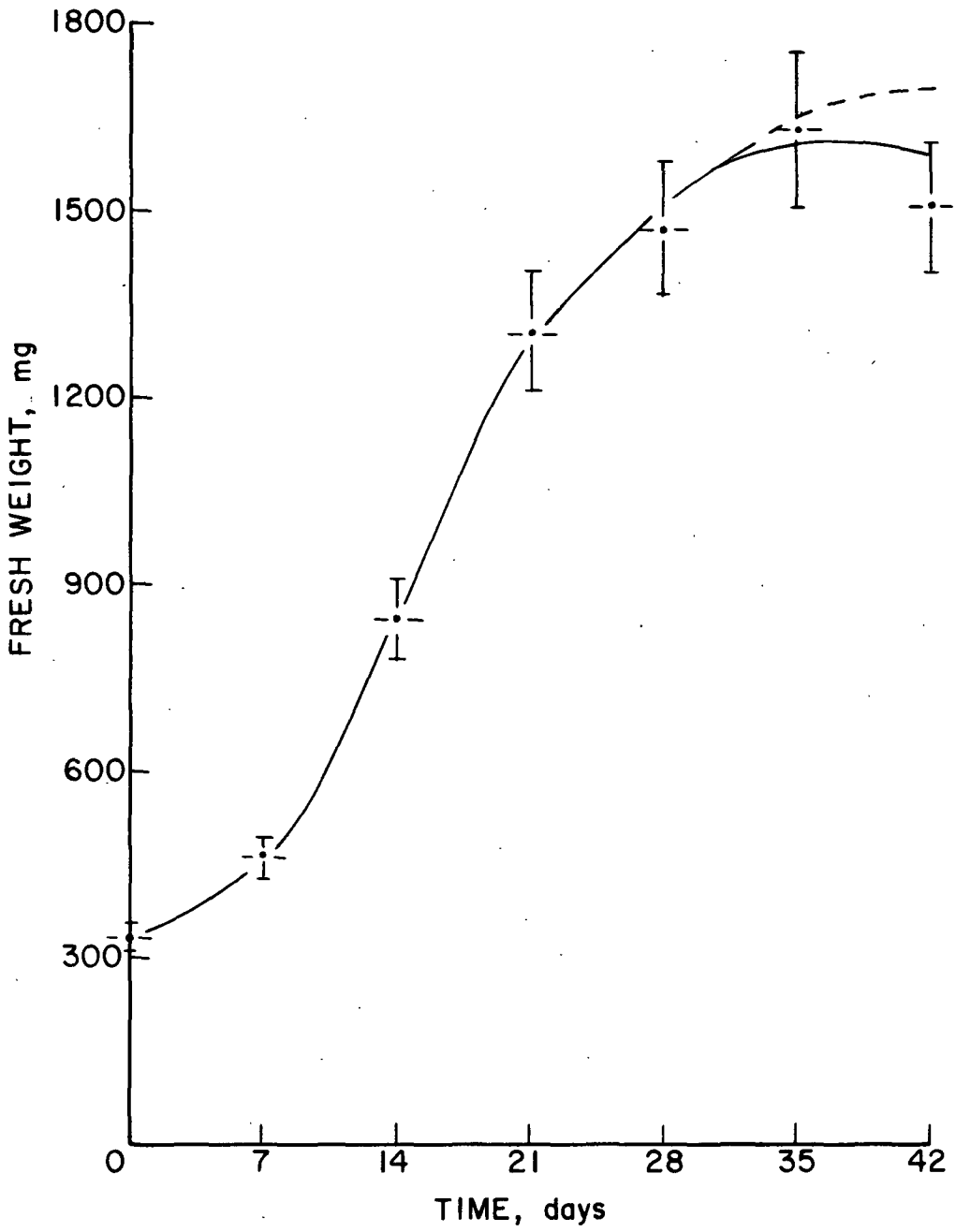


Figure 5. Cumulative Fresh Weight of Loblolly Pine Callus Following Subculture. Vertical Bars Represent Plus or Minus Standard Error of the Mean of Ten Determinations

percentage dry weight of the callus decreased from 5.0 to 3.6%. Water accounted for 98% of the callus net weight gain during this period. This result implies that cell expansion contributed more to callus growth than cell division.

CYCLIC AMP IN LOBLOLLY PINE CALLUS

The cyclic AMP concentration in loblolly pine callus was monitored over a six-week period following subculture with weekly assays using a cyclic AMP binding protein assay. One week after subculturing the callus, some pieces were obviously darker than others and these darker pieces were sampled for assay as a separate lot. On the basis of a dry weight: fresh weight ratio of 0.05, cyclic AMP concentrations in light-colored callus varied from 35 to 125 pmoles/g wet weight and in the darker callus, 24 to 345 pmoles/g wet weight, over the six-week period. These concentrations of cyclic AMP in loblolly pine callus fall within the range of cyclic AMP levels reported from other plant sources in Table I.

A direct relationship was observed between cyclic AMP levels and the rate of callus fresh-weight change (Fig. 6). The light-colored callus had cyclic AMP concentration maxima at two and five weeks from subculture while the maxima for the darker callus were at three and five weeks. The continued acceleration in callus fresh-weight growth during the third week, while the cyclic AMP level in the light-colored callus was falling, resulted from the fact that both light and dark callus pieces were weighed each week to determine the average rates of fresh-weight increase. The darker callus with increasing cyclic AMP therefore accounts for the continued acceleration of the average rate of fresh weight-gain during the third week. The rate of callus fresh-weight gain fell sharply during the fourth week when the cyclic AMP in lighter callus was already low and the level in darker callus was falling from the maximum at three weeks. An

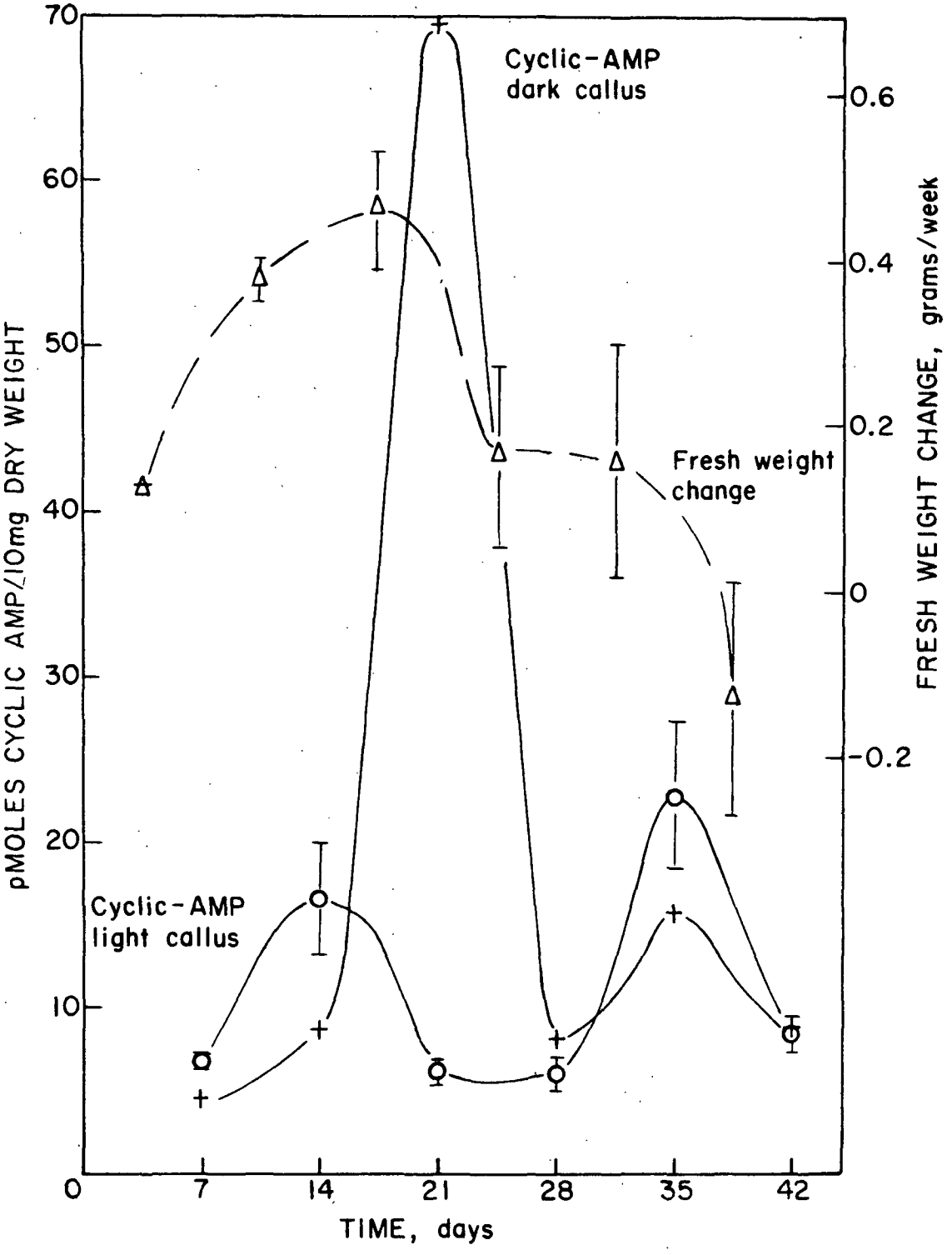


Figure 6. Fluctuation in Cyclic AMP Level and Fresh-Weight Growth of Loblolly Pine Callus with Time. Vertical Bars Represent Plus or Minus Standard Error of the Mean of Eight Determinations for Cyclic AMP and Ten Determinations of Fresh-Weight Change

acceleration in callus fresh-weight gain occurred again during the fifth week concomitant with the second cyclic AMP maximum for both light- and dark-colored callus.

Kamisaka, et al. (69) found that N^6,2'-O-dibutyryl cyclic AMP and 2,4-D acted synergistically in stimulating fresh-weight increase in Jerusalem artichoke tuber slices. If this relationship between cyclic AMP, auxin, and the rate of fresh-weight increase is the same for loblolly pine callus, the relationship between endogenous cyclic AMP levels and the rate of fresh-weight gain shown in Fig. 6 would be predicted, provided auxin was also present.

VERIFICATION OF THE CYCLIC AMP BINDING PROTEIN ASSAY RESPONSE

The saturation binding assay for cyclic AMP used in this work measures competition between cyclic AMP and [8-^{3}H]-cyclic AMP for binding sites on a cyclic AMP-dependent protein kinase isolated from bovine muscle. The validity of this assay for measuring cyclic AMP has been established for mammalian tissues and is currently one of the cyclic AMP assay methods of choice. The use of this or any other cyclic AMP assay, however, must be verified when assaying new kinds of tissues for the first time. Criticism of this and other cyclic AMP assay methods, as applied to plants, and the reported verification data were discussed in the Historical Review (32,33). Isolation of cyclic AMP in quantity from loblolly pine callus followed by its rigorous characterization was not practical because of the ultralow cyclic AMP concentration and the limited quantity of callus available. Rather, the objective here was to investigate thoroughly the observed assay response from loblolly pine callus extracts using the best available methods.

CHROMATOGRAPHY OF CALLUS EXTRACTS

The frozen, powdered callus was stirred with 6% trichloroacetic acid (TCA) to extract nucleotides and precipitate protein. Following TCA removal with diethyl ether and concentration, the extract was chromatographed on neutral alumina columns as described by White and Zenser (76). The light-yellow color of the extract was removed during chromatography. Elution volumes of a number of compounds reported by White and Zenser are shown in Appendix II, Table VII. Cyclic AMP recovery was estimated by recovery of [8-^{3}H]-cyclic AMP introduced in the extraction step and varied throughout the study from 17 to 60%, but did not vary more than ±6% from the mean recovery percentage in any experiment. This chromatographic method was the most effective of those investigated for routine use and gave the highest and most reproducible cyclic AMP recovery. Other methods examined included ion-exchange, thin-layer, and powdered cellulose column chromatography. The powdered cellulose column method was effective in separating TCA from cyclic AMP; however, ATP was chemically converted to cyclic AMP during the elution.

ASSAY RESPONSE EXAMINATION

Figure 7 shows the result of assaying a loblolly pine callus extract at several dilutions for cyclic AMP concentration using the cyclic AMP binding protein assay. The undiluted extract, relative concentration 1.0, was the equivalent of 1.23 g callus wet weight. The assay response did not diminish as expected with dilution until the extract was diluted at least 1:3 with assay buffer. The extract aliquot assayed at the 1:3 dilution was equivalent to 0.3 g callus wet weight. This result demonstrates that at an appropriate dilution, further dilution of the callus extract results in a proportional reduction in both the assay response and the amount of cyclic AMP indicated in the aliquot

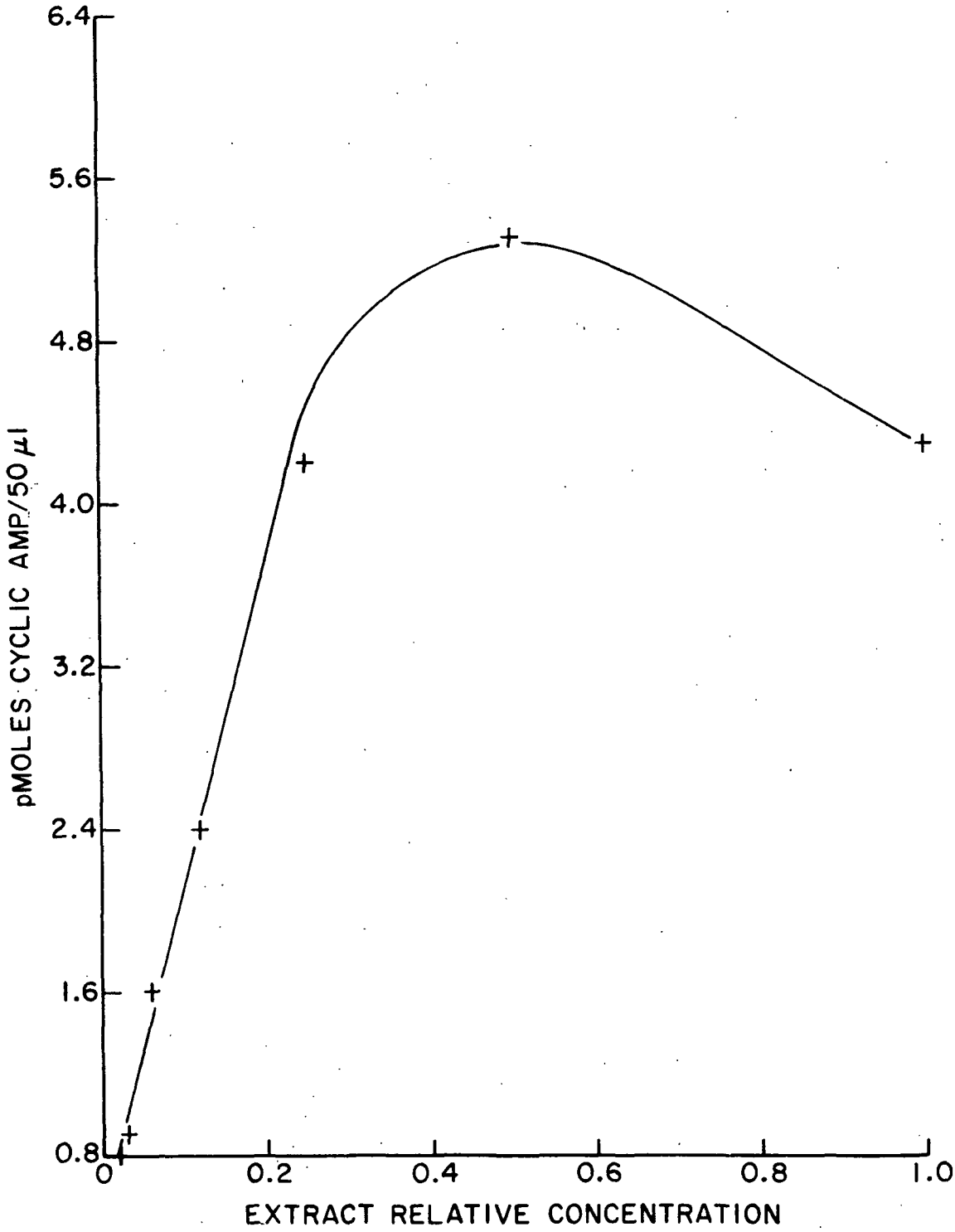


Figure 7. Cyclic AMP Concentration in Loblolly Pine Callus Extract Diluted with 50 mM tris-HCl (pH 7.5) Plus 1 mM $MgCl_2$ as Determined by Cyclic AMP Binding Protein Assay. Experimental Points are Single Determinations

assayed. Stated another way, this result establishes that some compound in the callus extract has the same affinity for cyclic AMP binding sites on the protein kinase as does authentic cyclic AMP. Its competition for those sites, however, may be observed only at proper extract dilution.

The nonlinear portion of the curve at higher relative extract concentration shows that some component of the partially purified callus extract stimulated cyclic AMP binding to the assay protein. The saturation binding assays use sufficient protein to bind 20-30% of the [^{3}H]-cyclic AMP available in the assay. The ratio $\underline{C}_o$, [^{3}H]-cyclic AMP bound in assay buffer only:$\underline{C}_x$, [^{3}H]-cyclic AMP bound in the presence of $\underline{x}$ pmoles cyclic AMP in the extract, is used to estimate cyclic AMP in the extract from the standard curve. In the presence of the activator, more cyclic AMP, both tritiated and nontritiated, was bound to the protein and the effect was to reduce the value of $\underline{C}_o/\underline{C}_x$, i.e., an inaccurately low assay of cyclic AMP. At proper extract dilution, the effect of the activator was diluted out and cyclic AMP could be assayed accurately.

Figure 8 shows the cross-reactivity of ATP and 3',5'-cyclic GMP in the cyclic AMP binding protein assay as reported by Amersham/Searle Corp. As shown, ATP does not cross-react with the assay at the concentrations tested and 0.1-$\mu\underline{M}$ cyclic GMP was required to give the same assay response as 1.0-n$\underline{M}$ cyclic AMP.

The results of investigating the cyclic AMP assay response from three different loblolly pine callus extracts are presented in Table II. After dilution, Extract B was assayed at the approximate concentration of cyclic AMP indicated by the dilution factor. Commercially prepared cyclic AMP was assayed accurately in the presence of Extract A and components of the cyclic-NPDE treatment. These are controls suggested by Gilman (43) for use with saturation binding assays for cyclic AMP. They demonstrate that cyclic AMP can be assayed

accurately in the presence of the callus extract and that some component of the extract competes equally with [^{3}H]-cyclic AMP for the assay protein binding sites.

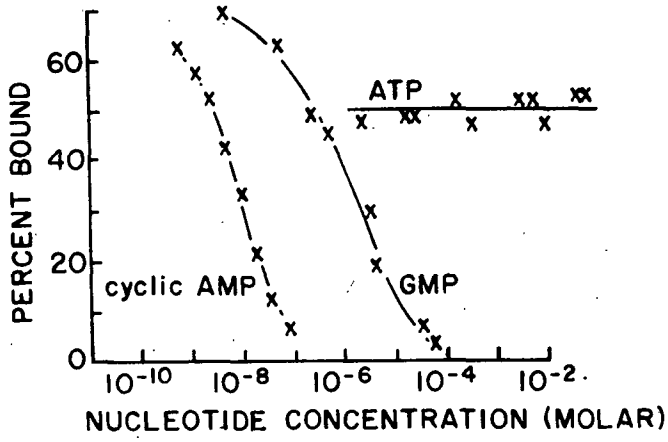


Figure 8. Cross-Reactivity of ATP and Cyclic GMP with Cyclic AMP Binding Protein, in the Presence of 4.5×10^{-9} M [^{3}H]-Cyclic AMP (83)

The assay response resulting from either authentic cyclic AMP or the callus extracts was reduced in a similar manner upon preincubation with 3',5'-cyclic NPDE. This enzyme was first isolated by Butcher and Sutherland (17) for use in characterizing adenosine 3',5'-cyclic monophosphate from biological sources. When purified by their method, the enzyme catalyzes the hydrolysis of 3',5'-cyclic nucleotides only. This enzyme is widely used to characterize cyclic AMP; however, commercial preparations are produced via abbreviated purification steps and contain, therefore, small amounts of other enzymes (see Appendix IV). The 3',5'-cyclic NPDE reaction conditions reported in Table II were sufficient for cyclic AMP hydrolysis in excess of the indicated amounts present. Additional 3',5'-cyclic NPDE reactions under conditions in which only a portion of the cyclic AMP indicated in the extract could be hydrolyzed were required to prove that the reduction in assay response occurred at a rate consistent with specific cyclic AMP hydrolysis.

TABLE II

VERIFICATION OF ADENOSINE 3',5'-CYCLIC MONOPHOSPHATE BINDING PROTEIN ASSAY RESPONSE FROM LOBLOLLY PINE CALLUS EXTRACTS

Treatment	Cyclic AMP,[c] pmoles
Extract A	26.2
Extract A + cyclic NPDE[a]	1.3
Extract A (+ 4.0 pmoles cyclic AMP after cyclic-NPDE treatment[a])	5.4
12.0 pmoles cyclic AMP + PDE[a]	1.1
Extract B	14.8
Extract B diluted 1:1	8.7
Extract C	3.3
Extract C + cyclic NPDE[b]	1.3

[a] PDE treatment was 0.2-ml callus extract incubated with 0.05 unit 3',5'-cyclic nucleotide phosphodiesterase, 33.2 μM tris and 1.8 μM $MgSO_4$ at pH 7.5, 30°C for 1 hr in a total volume of 0.65 ml.

[b] PDE treatment same as above except 0.013 unit 3',5'-cyclic nucleotide phosphodiesterase was in the reaction mixture and total reaction volume was 1.0 ml.

[c] Values from standard curve as described in Methods and Materials.

The results of 3',5'-cyclic NPDE-catalyzed hydrolysis of both authentic cyclic AMP and that in loblolly pine callus extracts are shown in Fig. 9. The 3',5'-cyclic NPDE concentration was 0.028 mg protein in 250 μl total volume. Callus extracts used in these reactions accounted for 200 μl of the total and were the 0.25 and 0.125 extract relative concentrations shown in Fig. 7. These reactions contained 17.2 and 10.0 pmoles of apparent cyclic AMP, respectively.

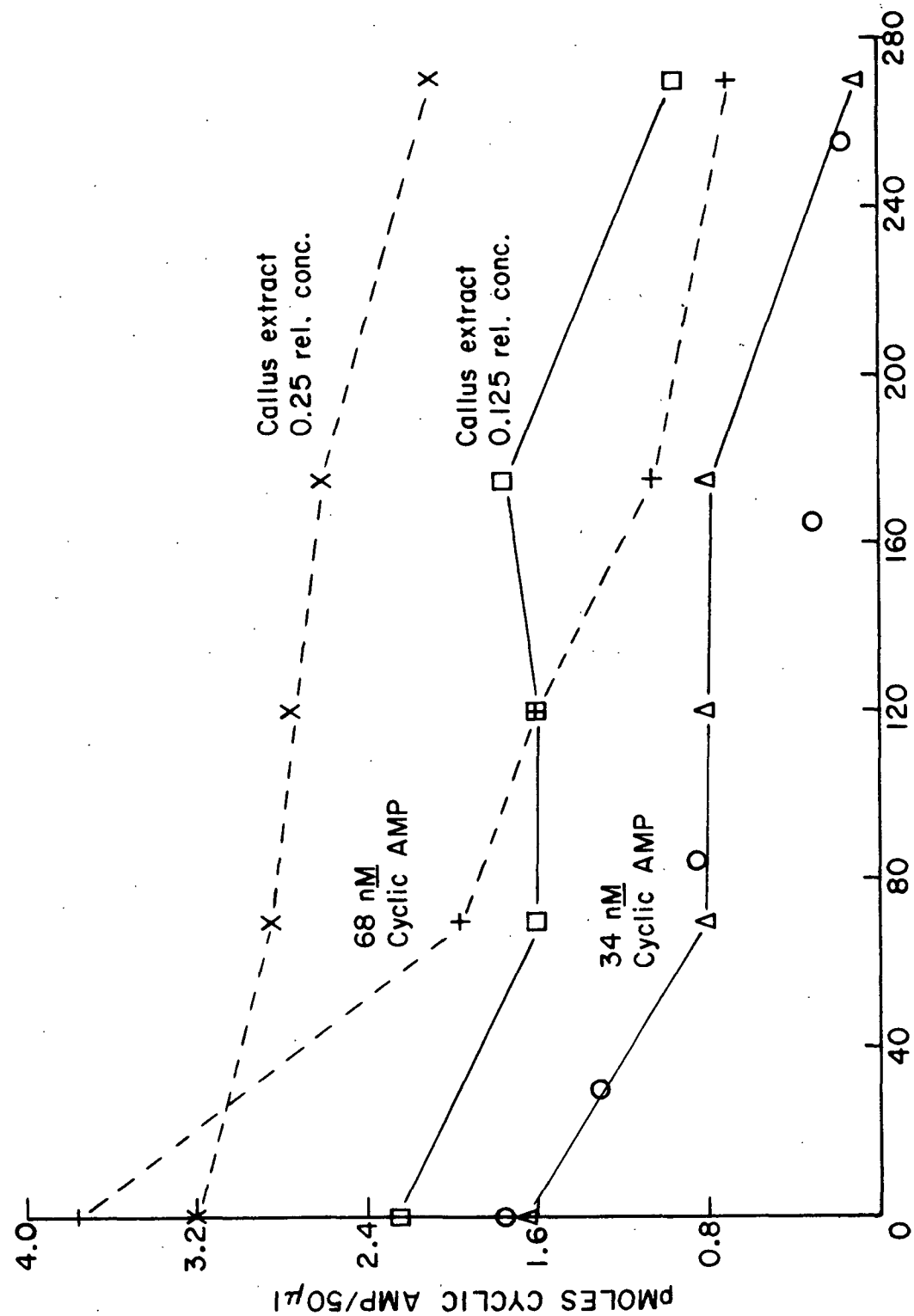


Figure 9. Change in Cyclic AMP with Time During Treatment with 3',5'-Cyclic Nucleotide Phosphodiesterase. 3',5'-Cyclic NPDE Concentration was 0.013 Unit/250 µl. Reactions Were Monitored by the Binding Protein Assay and Experimental Points are Averages of Duplicate Determinations

The decrease in assay response with time occurred at a slower rate for the callus extract of 0.25 relative concentration than was observed for cyclic AMP of approximately the same concentration. At the higher dilution, however, the reduction in the assay response from the callus extract approximately paralleled that of known cyclic AMP at a similar concentration. When the callus extract at 0.25 relative concentration was incubated with assay buffer in the absence of cyclic NPDE, the assay response did not decrease with time.

The 3',5'-cyclic NPDE activity appears to have been partially inhibited at the higher extract concentration. Wood, et al. (53) found in Vinca rosea tissue a nucleotide that inhibits cyclic NPDE from plants and animals. This nucleotide, cytokinesin I, has glucose as the sugar moiety and is the only other natural plant component known to inhibit cyclic NPDE of animal origin.

The Michaelis Equation (1) was used to calculate the expected hydrolysis of 3',5'-cyclic AMP with time under the reaction conditions used.

$$dy/dt = V_m(S_o - y)/(K_m + S_o - y) \qquad (1)$$

where

V_m = maximal reaction velocity per mg protein

K_m = substrate concentration giving 1/2 V_m

S_o = initial substrate concentration

y = concentration of substrate reacted at time t

t = reaction time

This equation assumes that S_o does not saturate the enzyme and that all changes in the reaction velocity, dy/dt, are the result of reductions in substrate available to the enzyme (84). This equation in integrated form (Equation 2) was used to estimate cyclic AMP hydrolysis with time. The solid circles in

Fig. 9 are calculated amounts of cyclic AMP remaining at specific times using Equation (2).

$$V_m t = \int_o^y (S_o - y + K_m)/(S_o - y) \cdot dy = y + K_m \ln S_o/(S_o - y) \qquad (2)$$

where

$\underline{S_o}$ = 34-n$\underline{M}$ cyclic AMP

$\underline{K_m}$ = 0.1 m$\underline{M}$

$\underline{V_m}$ = 0.45 µmole 3',5'-cyclic AMP hydrolyzed per mg protein per min, pH 7.5, 30°C

During the first 70 seconds of reaction, the degradation of commercial cyclic AMP and that estimated from the Michaelis equation indicated 3.5 and 3.6-pmoles cyclic AMP hydrolyzed, respectively. In this initial reaction interval, the assay response from the loblolly callus extract was decreased an amount equivalent to 2.5-pmoles cyclic AMP.

Based on the alumina chromatography and decreased assay response from the callus extract with excess 3',5'-cyclic NPDE, the list of compounds most likely responsible for the assay response included: 3',5'-cyclic AMP, 2',3'-cyclic AMP, 3',5'-cyclic GMP, and 3',5'-cyclic CMP. Of these cyclic nucleotides, cytidine 3',5'-cyclic monophosphate is as yet unknown naturally in living systems. Drummond and Perrott-Yee (85) reported 3',5'-cyclic NPDE to be ineffective in catalyzing 3',5'-cyclic CMP hydrolysis. Gilman (43) found 30-µ$\underline{M}$ 3',5'-cyclic CMP to be required for 50% inhibition of 40-n$\underline{M}$ cyclic AMP binding by the assay protein, i.e., 3',5'-cyclic CMP has a very low affinity for cyclic AMP binding sites on the assay protein. It was established by assaying loblolly callus extracts at many dilutions that the extract factor responsible for the assay response had the same affinity for cyclic AMP binding sites as authentic cyclic

AMP. It is unlikely, therefore, that the cyclic AMP binding protein assay response from loblolly pine callus results from 3',5'-cyclic CMP.

Sigma Chemical Co. reported that the V_m for 2',3'-cyclic nucleotide phosphodiesterase activity per milligram protein in the 3',5'-cyclic NPDE preparation used in this work was 0.006 times the V_m of 3',5'-cyclic AMP phosphodiesterase activity. The 2',3'-cyclic AMP degradable under the reaction conditions used was insignificant, and, therefore, 2',3'-cyclic AMP could not be responsible for the assay response from the callus extract.

Beavo, et al. (86) reported that the rate of 3',5'-cyclic GMP hydrolysis was several times faster than cyclic AMP hydrolysis when 1 μM substrate levels were used with this enzyme. Comparison of the rates of hydrolysis of these two cyclic nucleotides using similar reaction conditions to those reported in Fig. 9 indicated that 40-nM cyclic GMP was hydrolyzed at least 2.25 times the rate of 40-nM cyclic AMP hydrolysis in the first 30 seconds (Fig. 10). All results presented in this section indicate that 3',5'-cyclic AMP was responsible for the cyclic AMP assay response from loblolly pine callus extracts.

ENZYMES WHICH REGULATE CYCLIC AMP LEVELS

ADENYL CYCLASE

Reactions and enzymatic catalysts which regulate cyclic AMP concentration in animals and microorganisms were illustrated in Fig. 1. The presence of adenyl cyclase activity in loblolly pine callus was investigated with whole callus cells as well as with the pellet and supernatant resulting from centrifuging a callus homogenate at 600 × g. The binding protein assay for cyclic AMP was used to monitor cyclic AMP concentration with incubation time.

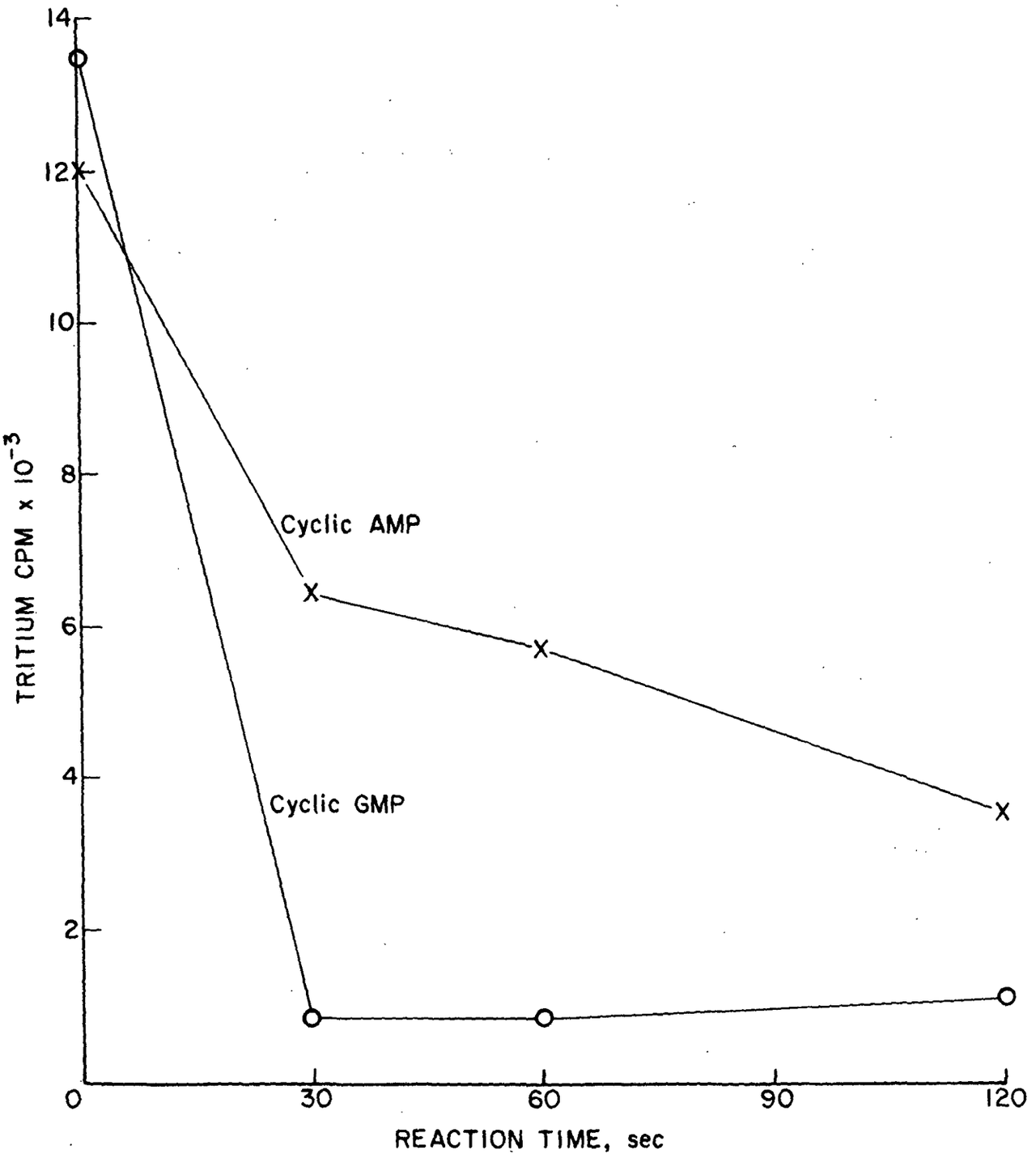


Figure 10. 40-nM 3',5'-Cyclic Nucleotide Hydrolysis Catalyzed by 3',5'-Cyclic Nucleotide Phosphodiesterase. Experimental Points are Averages of Duplicate Determinations

It was found that no net accumulation of cyclic AMP could be observed unless sodium fluoride was present in the incubation medium (Fig. 11). Sodium fluoride had been observed earlier in this study to inhibit cyclic AMP degradation in loblolly pine callus (Table IV, page 68) and its effect in the adenyl cyclase assay appears to have been the inhibition of product, cyclic AMP, hydrolysis. Giannattasio and Macchia (54) found 4-mM NaF to have no effect on adenyl cyclase activity in Jerusalem artichoke tubers. Sodium fluoride, however, is known to stimulate adenyl cyclase activity in broken cell preparations only from animal sources (3). Since the purpose of these investigations was to determine whether adenyl cyclase was present in the callus, the influence of sodium fluoride was not investigated further.

Cyclic AMP accumulated at the average rate of 0.033 pmole/mg protein/min during the first 30 minutes in the assay of the 600 × g supernatant. Experimental points in Fig. 11 are the averages of either 3 or 4 determinations and the standard error of the mean for each point is shown as a vertical bar. This cyclic AMP accumulation resulting from adenyl cyclase activity was undoubtedly a net increase in excess of that hydrolyzed. From 30 to 60 minutes of incubation time, the total amount of cyclic AMP decreased. This decrease in cyclic AMP might be expected when using crude extracts as an adenyl cyclase source and no ATP regenerating system. Such preparations probably contain ATPase activity as well as enzymes which catalyze cyclic AMP hydrolysis. Giannattasio and Macchia (54) reported a more rapid rate of cyclic AMP accumulation of 2.0 pmoles/mg protein/min for the first ten minutes of adenyl cyclase assay in a Jerusalem artichoke homogenate.

The 600 × g pellet had very little, if any, adenyl cyclase activity when examined under the same conditions as the supernatant. This fraction was

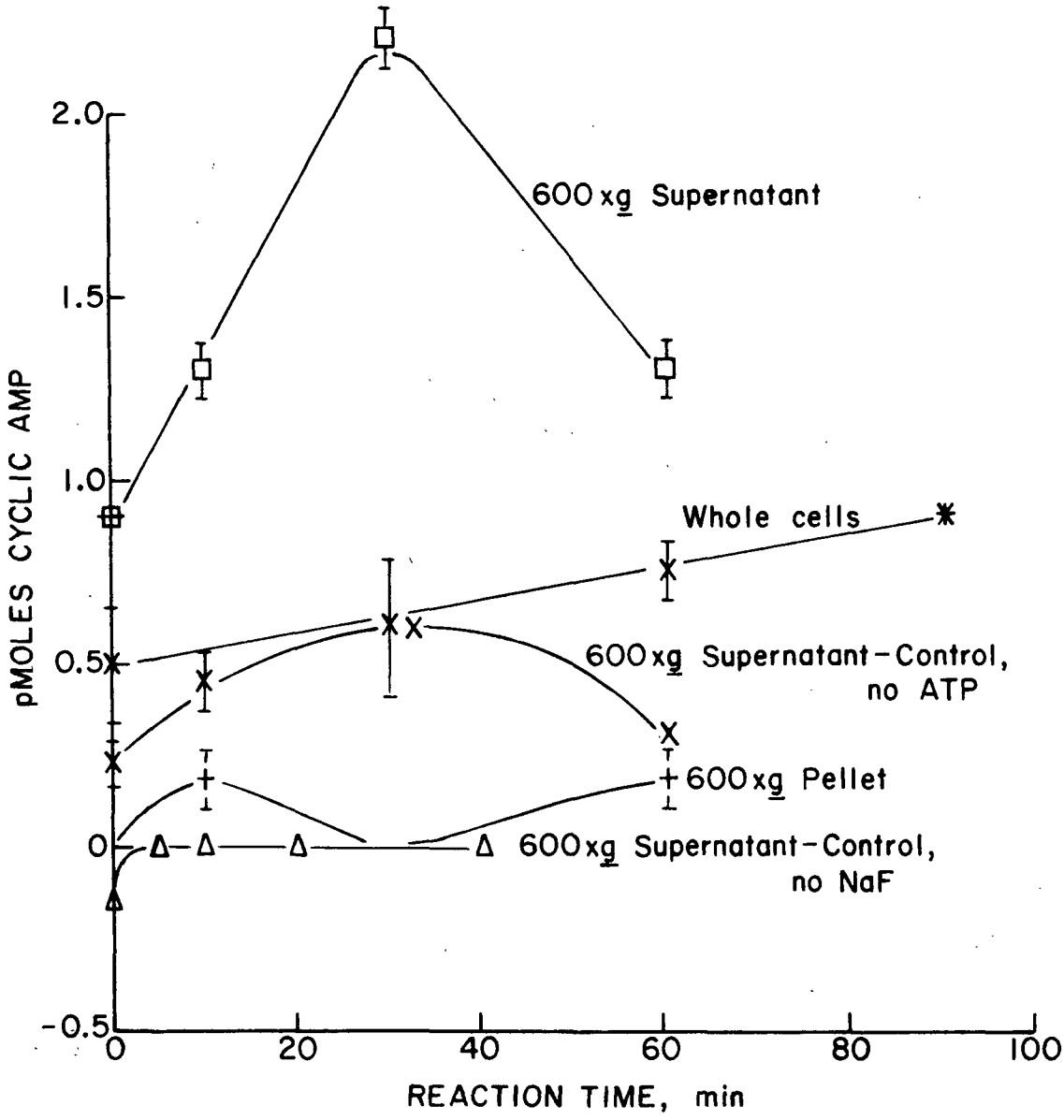


Figure 11. Cyclic AMP Accumulation with Time in Adenyl Cyclase Assay of Loblolly Pine Callus. Vertical Bars Represent Plus or Minus Standard Error of the Mean of Three Experimental Samples and Four Control Samples. ATP and NaF are Present in all Assays Unless Otherwise Indicated

expected to contain broken cell walls and nuclei. A relatively small amount of cyclic AMP accumulated during the first 30 minutes of the 600 × g supernatant control incubation with exogenously supplied ATP omitted. Assuming that ATP is found naturally in the callus, this result might be expected. This assumption is supported by carbon-14 activity found in loblolly callus extracts, following callus incubation with [8-^{14}C]-adenine, which migrated with ATP and ADP following three stages of thin-layer chromatography (Appendix XI).

A very low rate of cyclic AMP accumulation was also observed in assays with whole callus cells. In this case, there was no net observable cyclic AMP degradation. Several things could have affected the observed rate of cyclic AMP accumulation with whole callus cells. These factors include: the rate of ATP incorporation into whole cells; the uptake of sodium fluoride by the callus; and the effect of the cellular organization on the rate of cyclic AMP hydrolysis.

Two additional methods were used to investigate adenyl cyclase activity in whole callus cells. The histochemical method of Reik, et al. (87) was used to localize adenyl cyclase activity within the cell. The method involves the use of lead nitrate to precipitate, in situ, pyrophosphoric acid liberated from ATP in the adenyl cyclase reaction. Nonspecific lead deposits and problems with tissue fixation made the results impossible to interpret. Attempts to identify lead deposits resulting from the adenyl cyclase reaction by reducing the rate of conversion of ATP to cyclic AMP with massive cyclic AMP additions gave non-reproducible results.

The third method used to investigate adenyl cyclase activity in whole callus cells was an attempt to synthesize [8-^{14}C]-cyclic AMP from [8-^{14}C]-adenine. The incubation method used was similar to those employed by Pollard (49), Janistyne (51), and Amrhein (32). After incubation, whole cell clumps

were collected by filtering the cell suspension through cheesecloth, the cells were frozen in liquid nitrogen, powdered, and extracted with 6% TCA. The TCA was removed by water-saturated ether extraction. The filtrate (incubation medium) was also saved for analysis. Following adsorption of nucleotides on charcoal and eluting them from the charcoal with a basic alcohol solution, the eluate was neutralized and chromatographed on neutral alumina. The column eluate containing cyclic AMP was concentrated and mixed with appropriate internal standards. The tissue extract prepared in this way and containing internal [8-^{3}H]-cyclic AMP standard was chromatographed on a Bio-Gel P-2 column at elevated temperature, conditions similar to those used by John, et al. (78). Similarly, the incubation medium was chromatographed through Bio-Gel P-2 but without alumina chromatography. The elution volumes for internal standards (see Appendix III, Table XII) were established in separate runs with one or two compounds present.

Results of monitoring the eluates for carbon-14 and tritium as well as UV transmission at 254 nm are shown in Fig. 12 and 13. The tissue extract had no carbon-14 activity peak in the 3',5'-cyclic AMP elution region. The major carbon-14 peak corresponded to the elution volume for adenine, the starting material, and a carbon-14 activity peak also eluted with the adenosine standard. The carbon-14 peak eluting just after 3',5'-cyclic AMP was also present in the control incubation of [^{14}C]-adenine alone in phosphate buffer and, therefore, was not a result of callus biological activity. Another very small carbon-14 peak eluted at the same volume as 2',3'-cyclic AMP. No net [^{14}C]-cyclic AMP synthesis from [8-^{14}C]-adenine by the callus was demonstrated.

The incubation medium, when chromatographed on the Bio-Gel P-2 column had carbon-14 activity peaks which eluted at the same volumes as ADP, 5'-AMP,

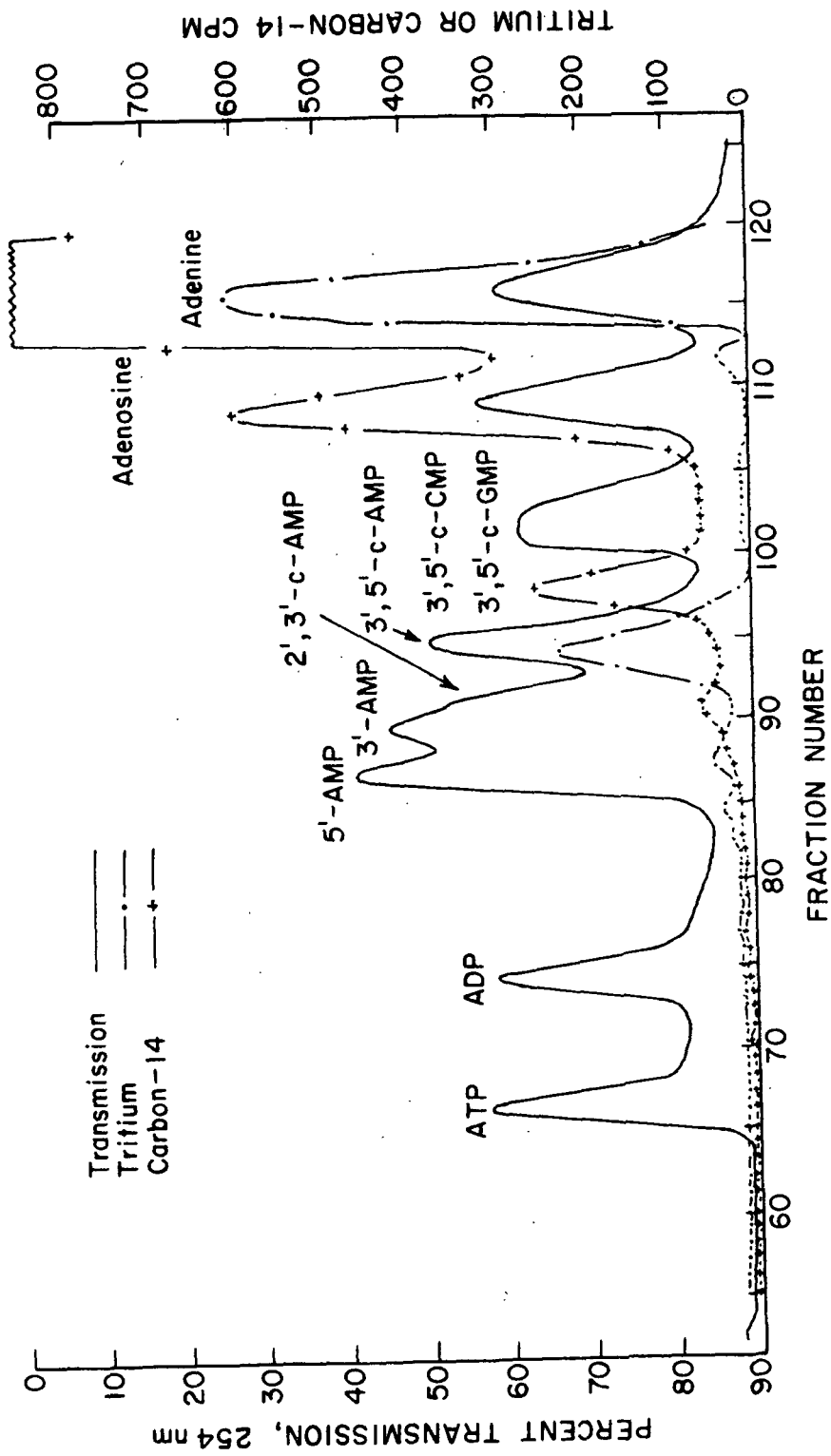


Figure 12. High Temperature Bio-Gel P-2 Chromatography of Loblolly Pine Callus Extract Following Callus Incubation with [8-^{14}C]-Adenine. Experimental Points are Single Determinations

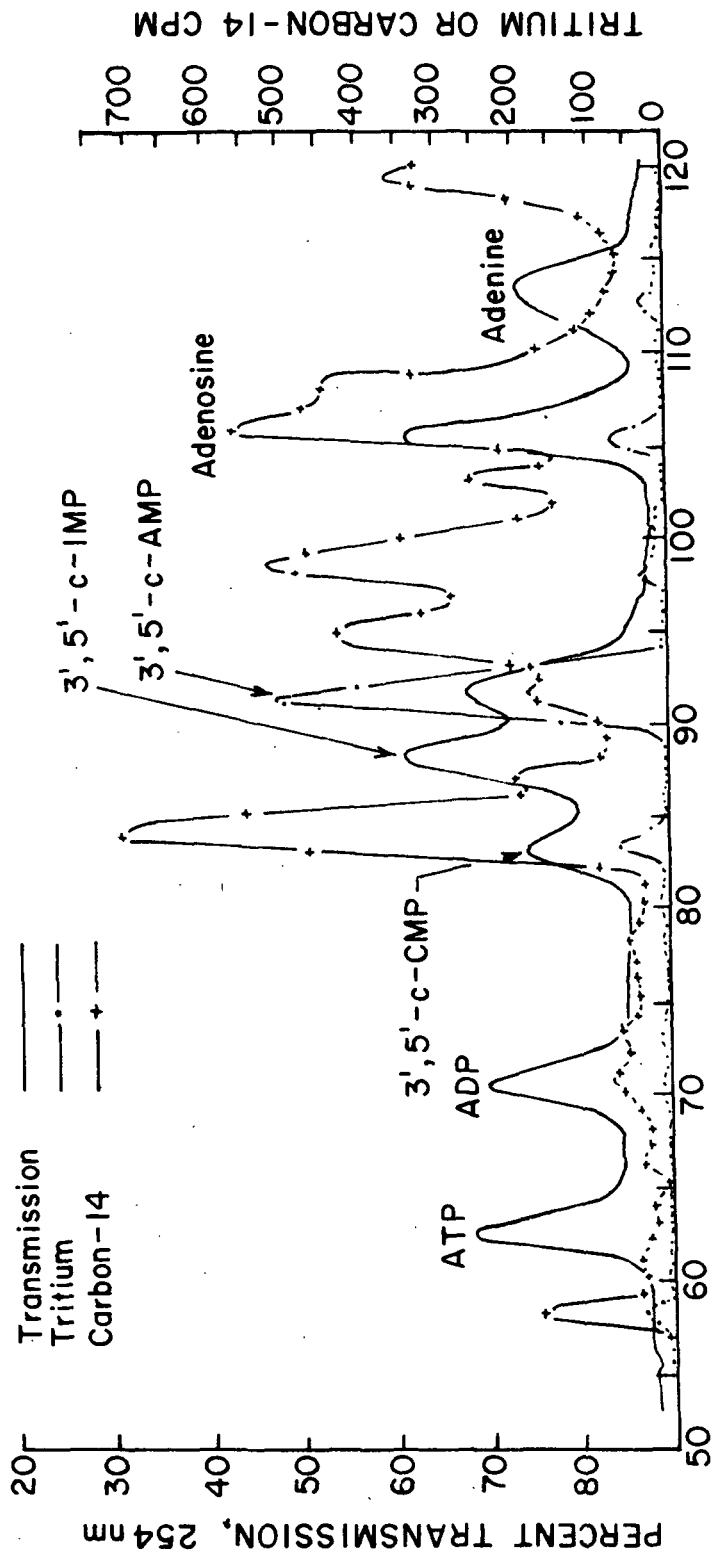


Figure 13. High Temperature Bio-Gel P-2 Chromatography of Carbon-14 Products in the Incubation Medium Following [8-^{14}C]-Adenine Incubation with Loblolly Pine Callus. Experimental Points are Single Determinations

3'-AMP, 3',5'-cyclic AMP, 3',5'-cyclic CMP, 3',5'-cyclic GMP, and adenosine. The carbon-14 count in the 3',5'-cyclic AMP region was a small shoulder on the front of the larger unknown carbon-14 peak which followed. The total carbon-14 count present in the 3',5'-cyclic AMP region was not sufficient for recovery and further investigation. See Appendix XII for simultaneous carbon-14 and tritium cpm calculations.

ENZYMES CATALYZING CYCLIC AMP DEGRADATION IN VIVO

Figure 14 shows the results of incubating [8-^{3}H]-cyclic AMP with whole callus cells from loblolly pine and separating the products by thin-layer chromatography. The major product separated resulting from [8-^{3}H]-cyclic AMP degradation was [8-^{3}H]-adenosine. [8-^{3}H]-Adenine increased with time at a very low rate, while [8-^{3}H]-AMP was present at a low and constant level throughout the incubation. A lag period of at least 15 minutes was observed in [8-^{3}H]-cyclic AMP degradation and in the appearance of [8-^{3}H]-adenosine. This lag period was interpreted as the time required for [8-^{3}H]-cyclic AMP uptake into the cell. Giannattasio, et al. (59) and Gordon, et al. (60) have likewise reported lag periods in cyclic AMP degradation in the presence of whole cells of Jerusalem artichoke tuber tissue and maize coleoptiles. Brewin and Northcote (38), however, found that whole soybean cells degraded cyclic AMP without an induction period. Results herein reported with loblolly pine callus indicated that cyclic AMP entered the tissue before being hydrolyzed.

The finding that adenosine was the major product isolated from cyclic AMP degradation by whole callus cells was consistent with other reports of cyclic AMP degradation in plants (38,53,54,56-58). Lin and Varner (58) separated cyclic NPDE and 3'-nucleotidase activities and demonstrated that these two

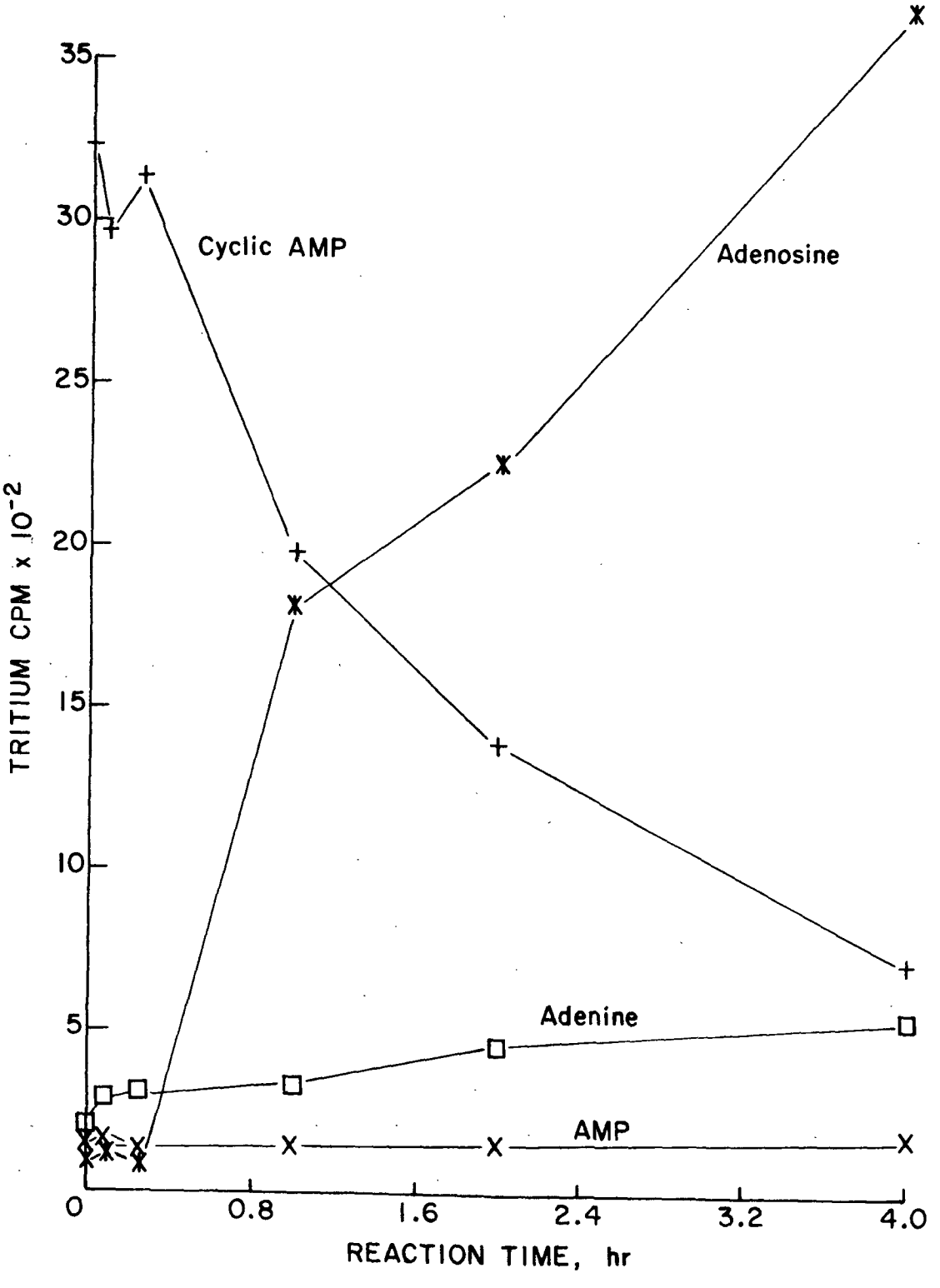


Figure 14. [8-^{3}H]-Cyclic AMP Degradation with Time by Loblolly Pine Callus Whole Cells. Experimental Points are Averages of Duplicate Determinations

enzymes were responsible for adenosine accumulation from cyclic AMP hydrolysis. They reported 3',5'-cyclic AMP to be hydrolyzed to 3'-AMP and 5'-AMP (7:1) while 2',3'-cyclic AMP was hydrolyzed to 3'-AMP only. The result of trapping [^{3}H]-AMP from [^{3}H]-cyclic AMP hydrolyzed by the crude loblolly pine callus homogenate and separated by TLC indicated that 3'-AMP and 5'-AMP were present (3:1).

CYCLIC AMP HYDROLYSIS IN CALLUS HOMOGENATE FRACTIONS

These investigations were undertaken to determine the approximate location of cyclic AMP degrading enzymes within the callus cell. The results of [8-^{3}H]-cyclic AMP degradative activity in different centrifugation fractions from callus homogenates are presented in Table III. Seventy-seven percent of the [8-^{3}H]-adenosine produced per milligram protein in all reactions occurred in the 12,100 × g pellet fraction which also contained the 600 × g solids. Total tritium activity in this 12,100 × g pellet fraction was lower than that recovered in other fractions. Tritiated compounds appeared to have been bound to the solids in this fraction.

When a whole callus homogenate was first centrifuged at 600 × g and the resulting supernatant then recentrifuged at 12,100 × g, the degradative activity of this 12,100 × g pellet was similar to the case in which heavier solids were included. This time 70% of [8-^{3}H]-adenosine from all fractions was produced in the 12,100 × g pellet. In this case though, no binding of tritium to the pellet was observed. [8-^{3}H]-Cyclic AMP count was higher than in the first experiment, which indicated that [8-^{3}H]-cyclic AMP was being selectively bound to a component of the 600 × g pellet solids. These solids were expected to consist mainly of broken cell walls and nuclei. Most of the cyclic AMP degradative activity other than that found in the 12,100 × g pellet was in solution. These results

suggested that most of the cyclic AMP degradative activity was localized in the larger organelles such as the mitochondria and/or plastids.

TABLE III

[^{3}H] PRODUCTS RESULTING FROM INCUBATING [8-^{3}H]-CYCLIC AMP WITH CENTRIFUGE FRACTIONS FROM A LOBLOLLY PINE CALLUS HOMOGENATE FOR 20 MINUTES

Centrifuge Fraction	Compound	Net [^{3}H], cpm[c,d,e] counted	Net Total [^{3}H], cpm	Protein/[f] Reaction, mg	[^{3}H] Reacted/ mg Protein, cpm
12,100 × g Pellet	AMP	79	205	0.036	5,694
	Cyclic AMP	832	2163		
	Adenosine	361	939		26,083
12,100 × g Supernatant	AMP	13	34	0.056	607
	Cyclic AMP	2134	5548		
	Adenosine	107	278		4,964
100,000 × g Pellet[a]	AMP	43	112	0.034	3,294
	Cyclic AMP	2488	6469		
	Adenosine	(63)	(164)		(4,824)
100,000 × g Supernatant[b]	AMP	2	5	0.056	89
	Cyclic AMP	1951	4073		
	Adenosine	67	174		3,107

[a] Pellet resulting from recentrifuging the 12,100 × g supernatant.

[b] Supernatant resulting from recentrifuging the 12,100 × g supernatant.

[c] Background for tritium was 59 cpm.

[d] Conversion factor from net tritium cpm to net total tritium cpm was 2.6.

[e] At t = 0, AMP = 35 cpm; adenosine = 119 cpm.

[f] Protein determinations were by the biuret method (77).

The rate of cyclic AMP degradation with increasing amounts of the 12,000 × g pellet fraction at constant reaction volume was studied in 10-minute reactions. The reduction of [8-^{3}H]-cyclic AMP and the increase of [8-^{3}H]-adenosine with increasing amounts of the callus fraction are illustrated in Fig. 15. In the absence of substances interfering with the reaction, two molecules of enzyme

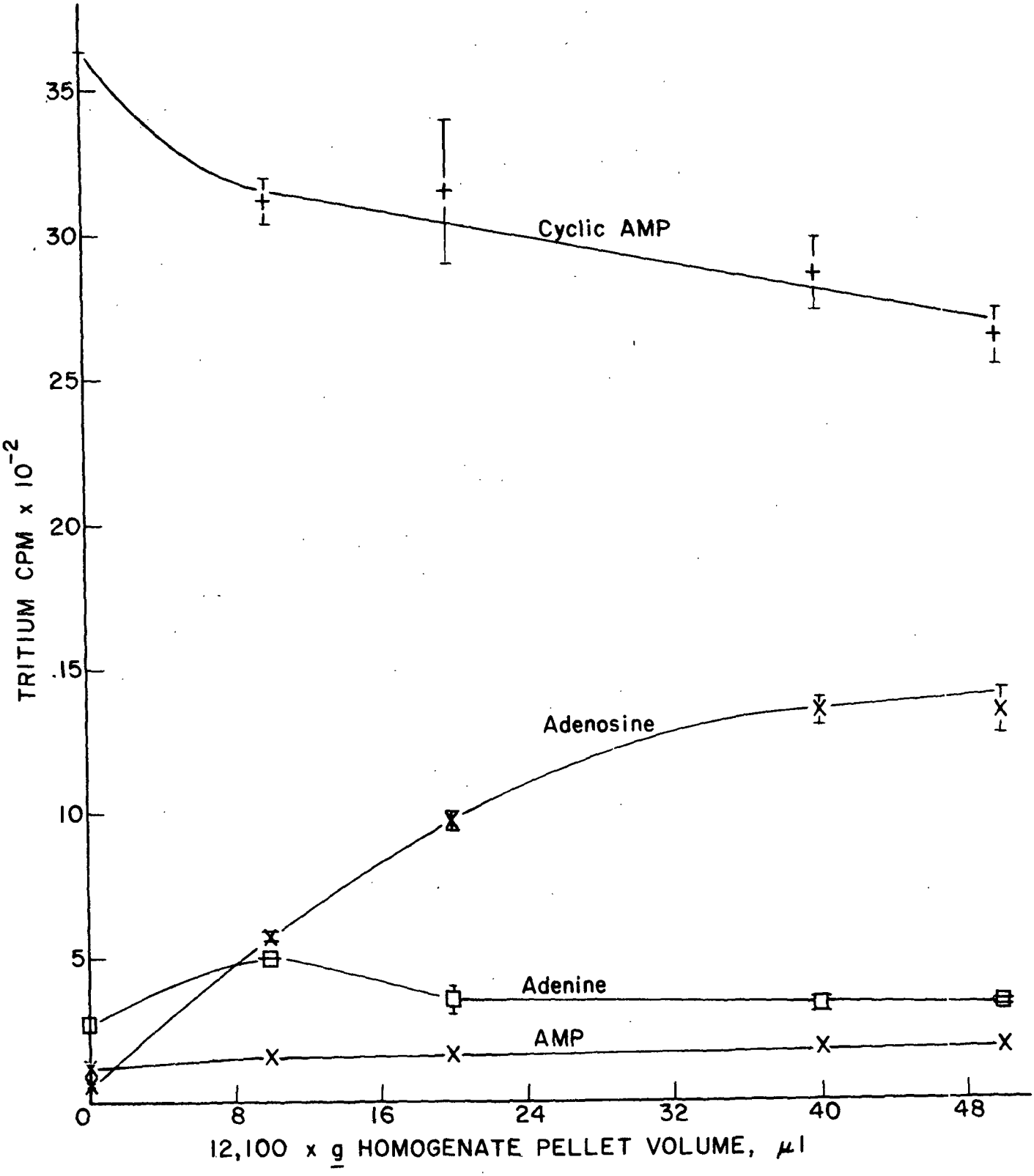


Figure 15. [8-^{3}H]-Cyclic AMP Degradation with Various Amounts of the 12,100 × g Pellet from a Loblolly Pine Callus Homogenate. Vertical Bars Represent Plus or Minus Standard Error of the Mean of Four Determinations

should react with twice as much substrate per unit time as one enzyme molecule. Therefore, the initial reaction velocity should be proportional to enzyme concentration so long as no inhibitors are present and the substrate levels are sufficient to saturate the enzyme. The results in Fig. 15 indicated that increasing the concentration of the 12,100 × g homogenate pellet in the [8-^{3}H]-cyclic AMP degradation reaction did not produce a proportional increase in reaction velocity. The purpose in this investigation of 12,100 × g homogenate pellet was to verify that the [8-^{3}H]-cyclic AMP degradation resulted from enzymatic activity in the pellet. A reaction rate dependence on pellet concentration was demonstrated but the reason for the nonlinear relationship was not investigated.

Inhibitors of Cyclic AMP Hydrolysis

Compounds known to inhibit cyclic AMP hydrolysis in other plants were tested on enzymes which catalyze cyclic AMP hydrolysis in loblolly pine callus. This work was done not only to investigate the similarities of the action of these compounds in loblolly pine callus and other plants, but also to find an inhibitor of cyclic AMP degradation to be used in the adenyl cyclase assay. Table IV shows the results of the action of some compounds on [^{3}H]-cyclic AMP degradation.

The most effective inhibitors of cyclic AMP degradation were 10-mM sodium fluoride and 1-mM monobasic potassium phosphate with 4-mM EDTA being somewhat less effective. Theophylline, 8 mM, did not inhibit cyclic AMP degradation. Other concentrations of these compounds were not tested. These results are consistent in most respects with the findings of other workers investigating cyclic AMP degrading enzymes in plants (38,54,56,58). The inhibition of cyclic AMP degradation with EDTA in loblolly pine callus was similar to the observations

of Vandepeute, et al. (56) with cyclic NPDE from barley seeds. The other workers reported little or no effect of EDTA on cyclic AMP hydrolysis by enzymes isolated from plant sources.

TABLE IV

EFFECTS OF SEVERAL COMPOUNDS ON CYCLIC AMP HYDROLYSIS BY THE 12,100 × g PELLET FROM A LOBLOLLY PINE CALLUS HOMOGENATE

Additive	Decrease in [^{3}H]-Cyclic AMP (% of Control)
Control-1 (none)[a]	100
4-mM EDTA[a]	82
8-mM Theophylline[a]	106
1-mM KH_2PO_4[a]	45
Control-2 (none)[b]	100
10-mM NaF[b]	10

[a][^{3}H]-Cyclic AMP disappearing from 0 to 10 minutes.

[b][^{3}H]-Cyclic AMP disappearing from 5 to 20 minutes.

5'-Nucleotidase Activity

To test for the presence of 5'-nucleotidase in loblolly callus, [8-^{14}C]-5'-AMP (specific activity 57.4 mCi/mmole) and nonradioactive 0.16-mM cyclic AMP were incubated with an aliquot of the 12,100 × g centrifuge pellet. The resulting carbon-14-labeled compounds in the reaction mixture at different reaction times were separated by TLC and the amounts determined by liquid scintillation counting as shown in Fig. 16. [8-^{14}C]-5'-AMP was hydrolyzed to [8-^{14}C]-adenosine at the average rate of 13.5 pmoles/min. [8-^{14}C]-Adenine accumulated at an average rate of 0.9 pmole/min. Since the accumulation of [8-^{14}C]-adenine was linear with time, [8-^{14}C]-5'-AMP as well as [8-^{14}C]-adenosine appeared to have been degraded to [8-^{14}C]-adenine by the callus

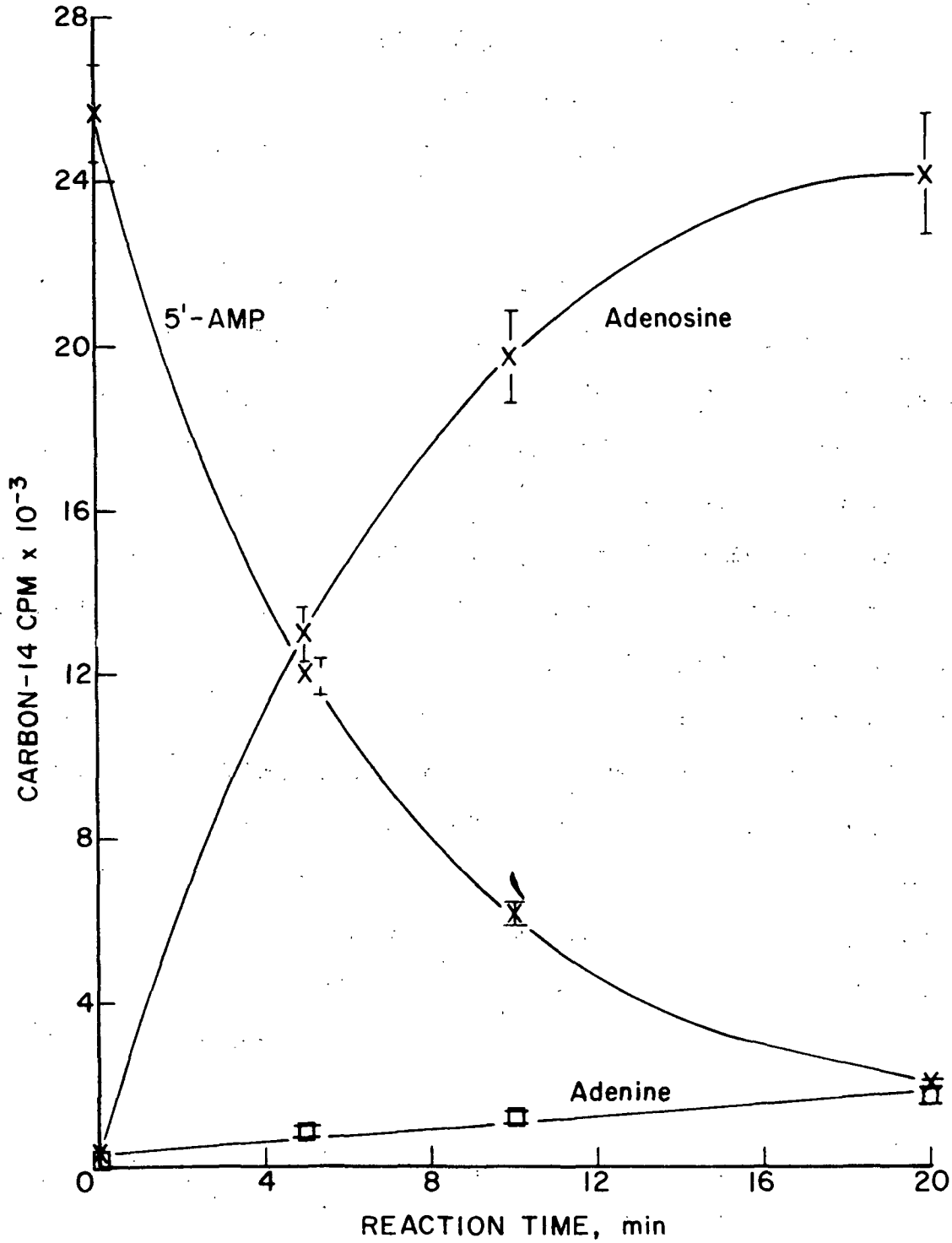


Figure 16. [8-^{14}C]-5'-AMP Degradation with Time by the 12,100 × g Pellet from a Loblolly Pine Callus Homogenate. Vertical Bars Represent Plus or Minus Standard Error of the Mean of Four Determinations

preparation. Reincubation of [8-^{14}C]-adenosine with the 12,100 × g pellet fraction from the homogenate confirmed that [8-^{14}C]-adenosine was being converted to [8-^{14}C]-adenine. Rees and Duncan (88) reported an adenyl-cleaving enzyme present in potato tubers which had the same activity for adenosine, AMP, ADP, and ATP.

Figure 17 shows that a linear relationship exists between the reaction time and ln (initial [8-^{14}C]-5'-AMP concentration/[8-^{14}C]-5'-AMP concentration at time t) for the first ten minutes of reaction. The slight deviation from a linear relationship in the 10 to 20 minute reaction interval probably resulted from a dilution of [8-^{14}C]-5'-AMP specific activity resulting from 5'-AMP arising from the hydrolysis of added unlabeled cyclic AMP. This plot indicates that in the presence of cyclic AMP, the 5'-nucleotidase exhibited first-order reaction kinetics. Figure 18 illustrates the reaction sequence of the hydrolysis of cyclic AMP in pea seedlings leading to adenosine proposed by Lin and Varner (58). The results of investigating cyclic AMP hydrolysis in loblolly pine callus were consistent with the reaction sequence they proposed. Since AMP levels were low and relatively constant in all cyclic AMP hydrolysis reactions observed, a steady-state approximation may be applied to AMP and the degradation rate of cyclic AMP described as its rate of hydrolysis to adenosine. The rate limiting step, then is the phosphodiesterase catalyzed hydrolysis of cyclic AMP to 3'-AMP or 5'-AMP.

EFFECTS OF EXOGENOUS AUXIN TREATMENTS ON CYCLIC AMP CONCENTRATION IN LOBLOLLY PINE CALLUS

A secondary objective of this thesis was to determine the effect, if any, of auxin on cyclic AMP concentration in loblolly pine callus. The effect of exogenously supplied auxin on cyclic AMP levels was studied with two differen-

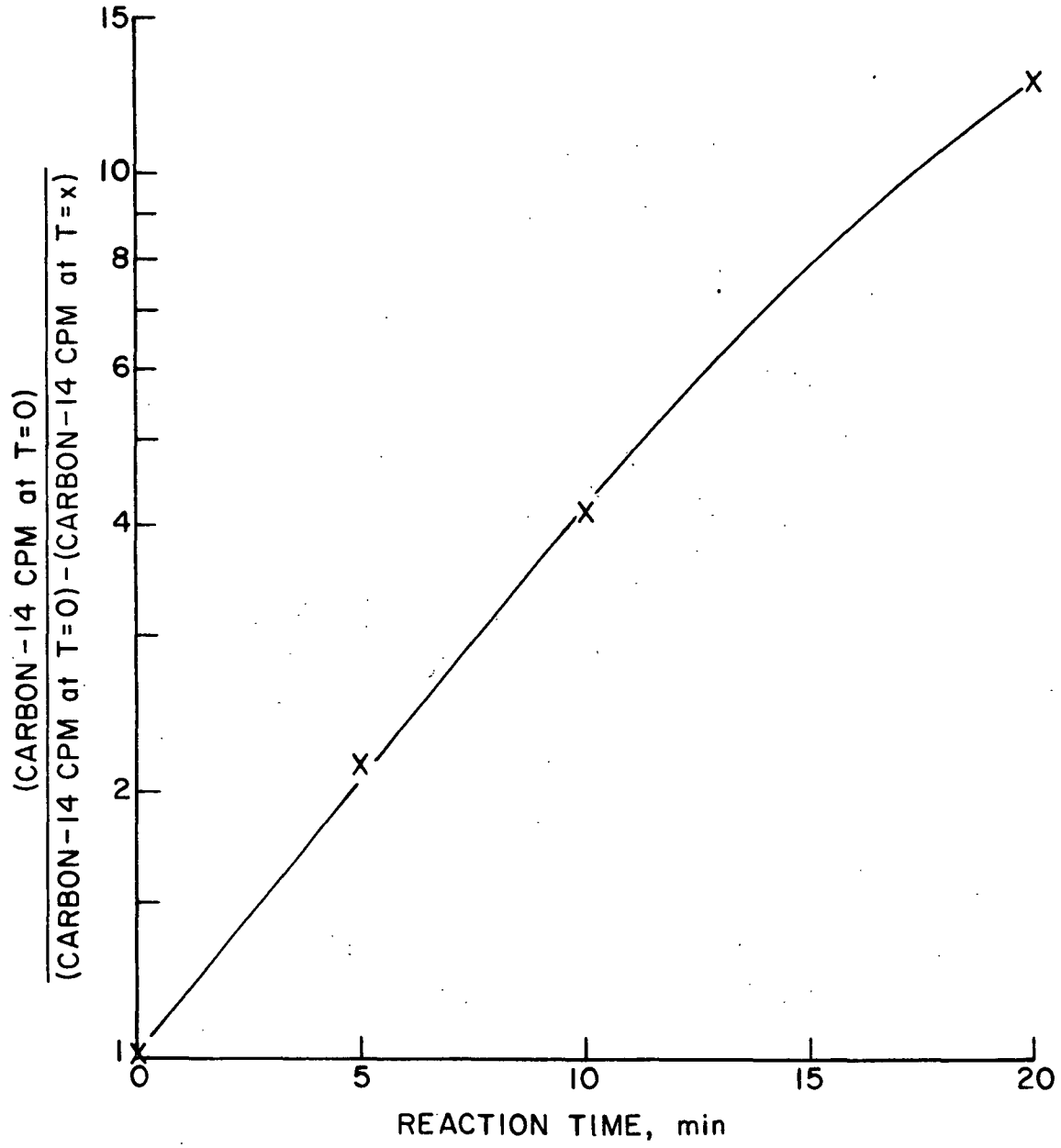


Figure 17. [8-^{14}C]-5'-AMP Degradation with Time by the 12,100 × g Pellet from a Loblolly Pine Callus Homogenate. Experimental Points are Averages of Four Determinations

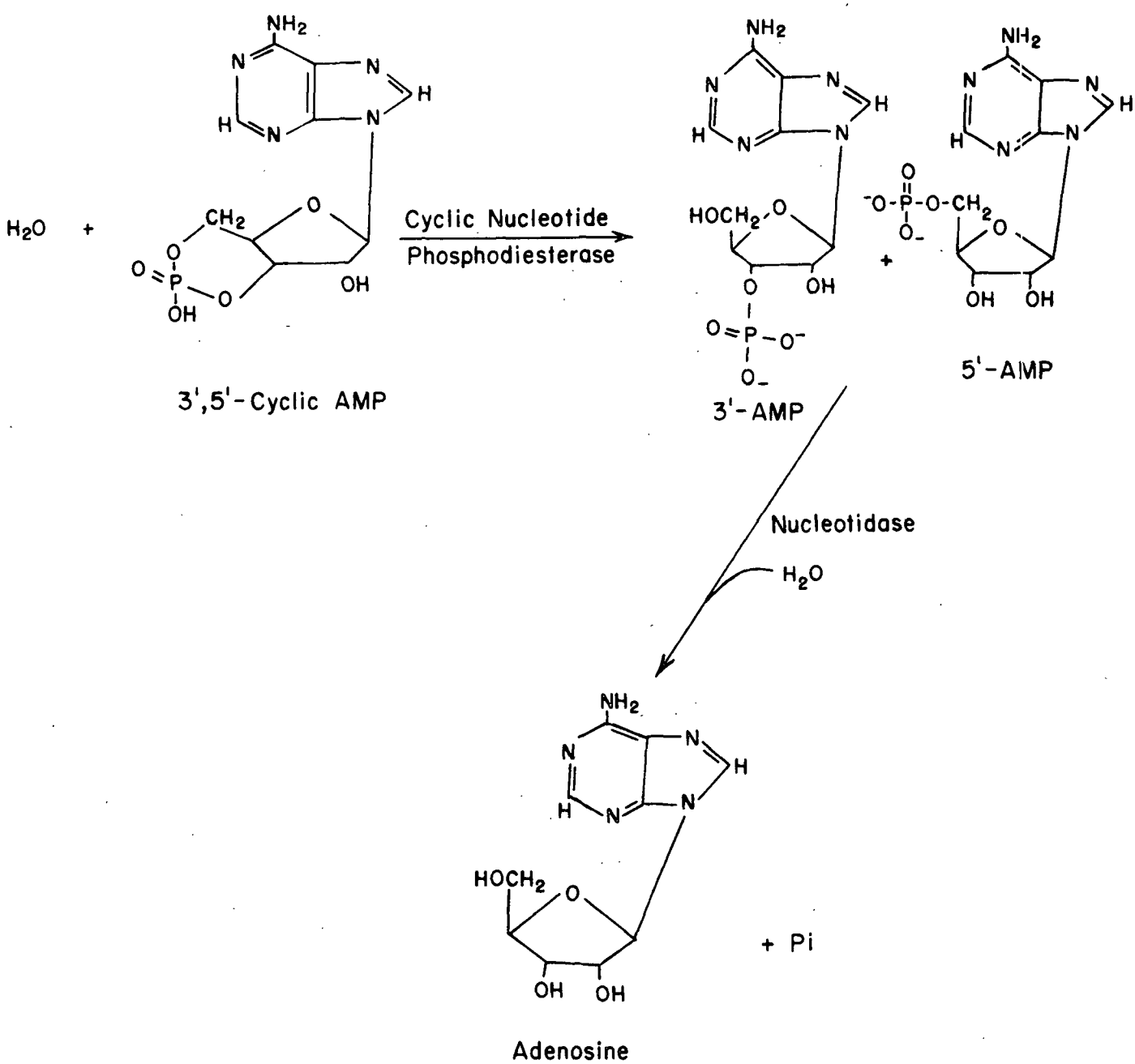


Figure 18. Reactions Proposed by Lin and Varner (58) for Cyclic AMP Hydrolysis in Pea Seedlings Resulting in Adenosine Formation

types of treatment: a) 2,4-D in aqueous solution was dropped on callus growing in place on agar medium and b) either 2,4-D or IAA were supplied as components of incubation mixtures in which cyclic AMP degradation with the 12,100 × g pellet fraction from a callus homogenate was assayed.

AUXIN EFFECT ON EXTRACTABLE CYCLIC AMP FROM CALLUS WHOLE CELLS

Figure 19 shows the results of cyclic AMP assays after dropping 2.3-μM 2,4-D, 23-μM 2,4-D, and double-distilled water on callus as it grew in the dark on agar. For at least 20 minutes after auxin treatment the amount of cyclic AMP that was extracted from callus cells differed from that of control cells. The lower 2,4-D concentration was the most effective in this action. One hour after treatment, no difference was observed in cyclic AMP levels between treatments. The observed reduction in extractable cyclic AMP may be occurring by any one or combination of three mechanisms including: a) cyclic AMP degradation; b) cyclic AMP secretion into the agar; and/or c) cyclic AMP binding to other substances not soluble in 6% TCA. The following study was designed to determine the effect of auxin on cyclic AMP degradation.

AUXIN EFFECT ON CYCLIC AMP LEVEL ASSOCIATED WITH CALLUS HOMOGENATE FRACTIONS

The results of incubating the 12,100 × g pellet fraction (see footnote, page 32) from a loblolly pine callus homogenate with [8-^{3}H]-cyclic AMP both in the presence of 2.3-μM 2,4-D and with no 2,4-D are shown in Fig. 20. Smaller amounts of all compounds were isolated by TLC at each reaction time from reactions containing 2,4-D than from the reactions without 2,4-D. Since the same total [8-^{3}H]-cyclic AMP was present in all reactions, the recovery of tritiated compounds was lower when 2,4-D was present in the reaction medium. Further

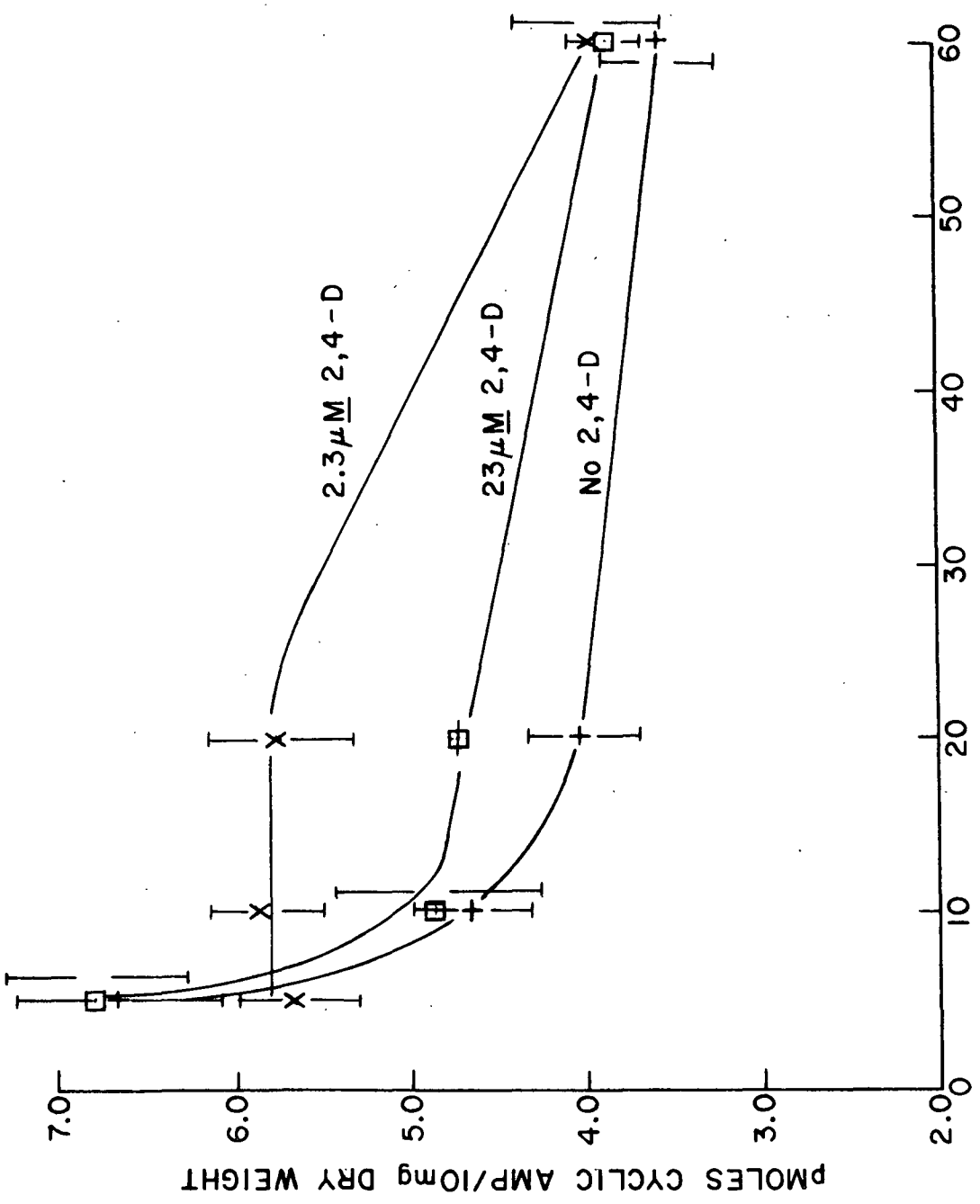


Figure 19. Variation of Cyclic AMP Concentration in Loblolly Pine Callus with Time Following Auxin Treatment of the Callus Growing on Agar. Vertical Bars Represent Plus or Minus Standard Error of the Mean of Four Determinations

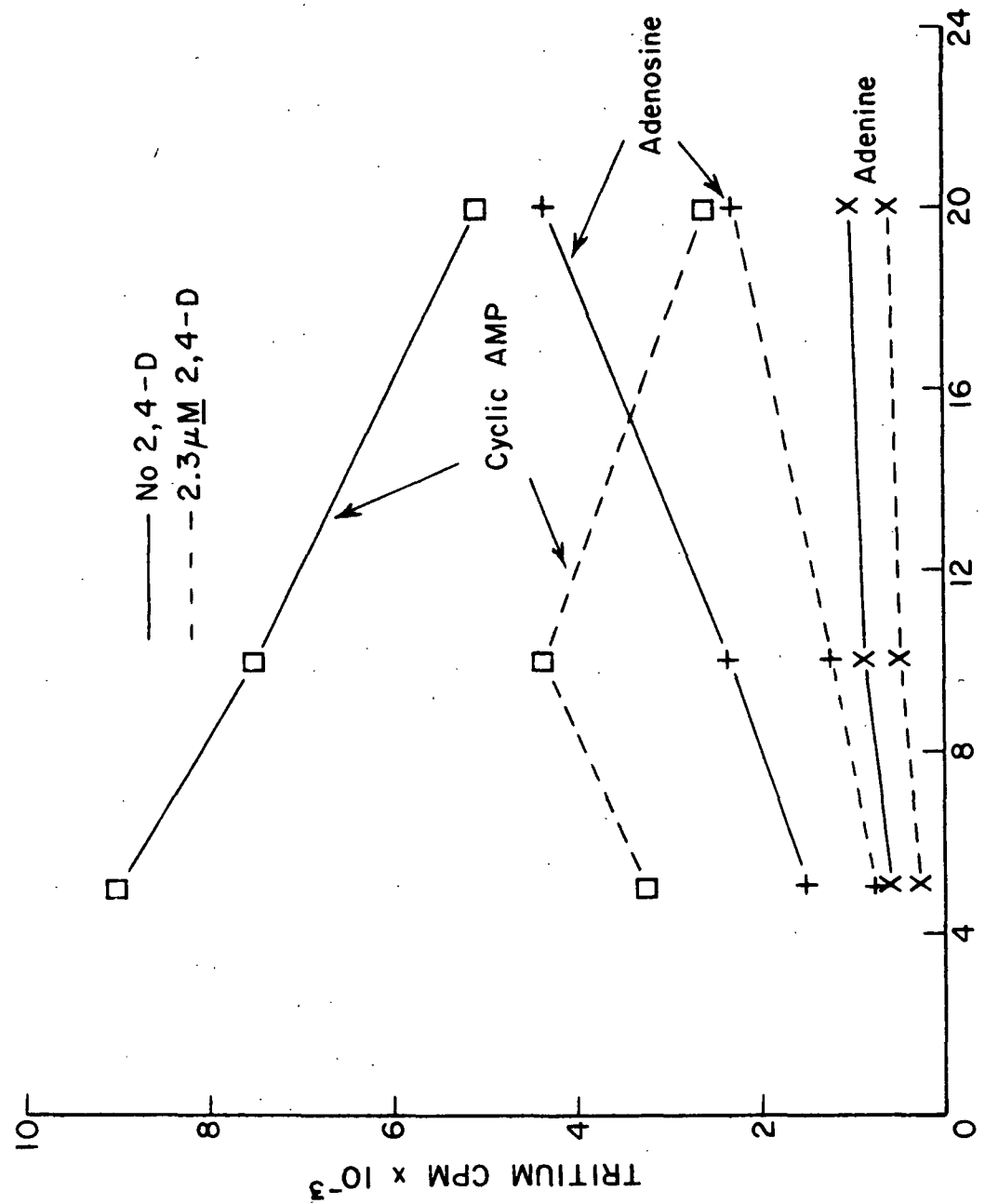


Figure 20. Effect of 2.3-μM 2,4-D on [8-^{3}H]-Cyclic AMP Degradation by the 12,100 × g Pellet from a Loblolly Pine Callus Homogenate. Experimental Points are Averages of Duplicate Determinations

investigations showed that [8-^{3}H]-adenine derivatives were being bound to a component of the 12,100 × g callus pellet fraction and that 85% of these compounds was released by treatment with TLC II developer, n-butanol-methanol-ethyl acetate-concentrated ammonium hydroxide (7:3:4:4) (Fig. 21). The binding of [8-^{3}H]-adenine derivatives was occurring in the absence of 2,4-D; however, this binding was enhanced by the presence of 2,4-D.

These observations of the effect of 2,4-D on cyclic AMP extractable from the callus homogenate appeared to be the reverse of the observations from dropping 2,4-D on whole callus growing on agar (Fig. 19). This difference could have resulted from the effect of grinding and examining only a fraction of the total callus. The callus used in these two studies was from the same subculture, but had grown for 28 days before the dropping test and 21 days before preparing and examining the homogenate.

The effect of auxin on [^{3}H]-cyclic AMP in solution in the presence of the 12,100 × g callus pellet fraction was repeated using loblolly pine callus which had grown 15 days since subculturing. Auxin was supplied as 2.3-μM 2,4-D and 10-μM IAA. Smaller differences in cyclic AMP disappearance with time were observed between treatments than in the preceding experiment (Fig. 22). Additionally, the auxin effect on [^{3}H]-cyclic AMP in solution was reversed from the previous experiment where callus 21 days from subculture was used. After five minutes reaction, the [8-^{3}H]-cyclic AMP in solution was about the same in all treatments. In the absence of auxin, the soluble [8-^{3}H]-cyclic AMP continued to decline linearly for 15 minutes. [8-^{3}H]-Cyclic AMP in the presence of 2,4-D was reduced at a slower, but almost linear rate for 20 minutes. During the first ten minutes of reaction, the IAA treatment was much like the 2,4-D treatment but, during the last ten minutes, was similar to the no-auxin treatment. This

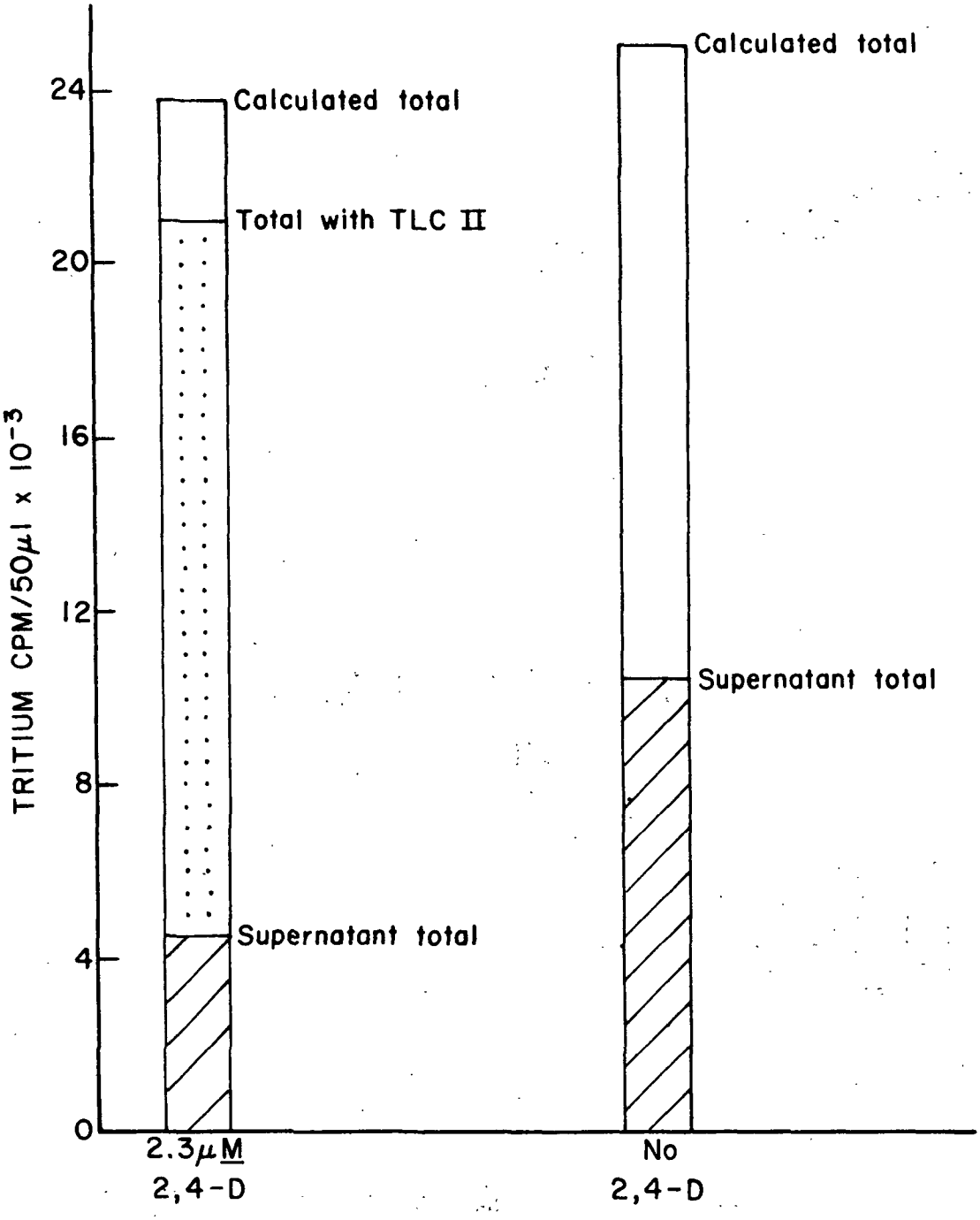


Figure 21. Tritium Activity Recovery After 5-Minute [8-^{3}H]-Cyclic AMP Incubation with 12,100 × g Pellet from Loblolly Pine Callus Homogenate and Tritium Activity Released by TLC II Developer

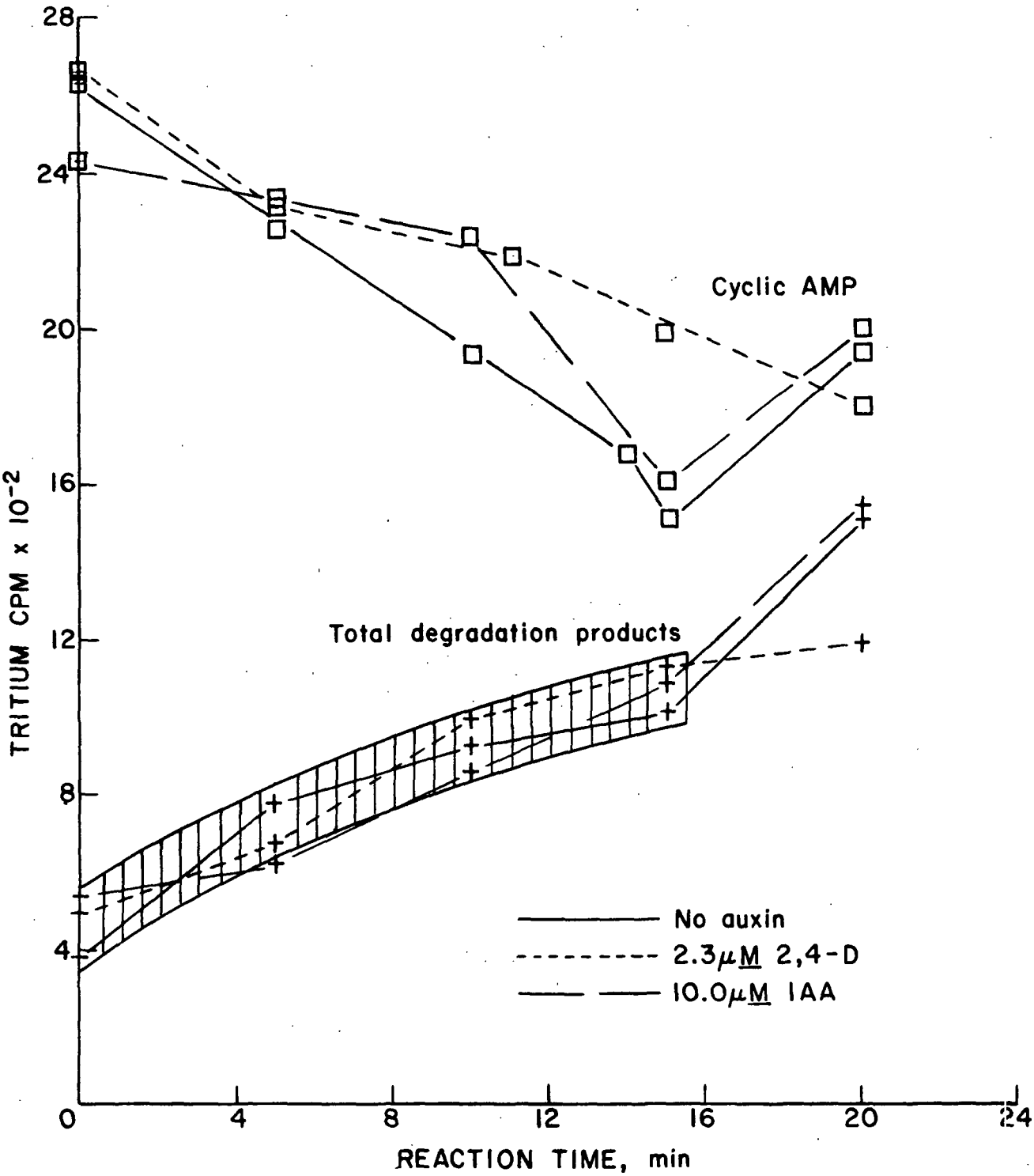


Figure 22. Effect of Auxins on [8-^{3}H]-Cyclic AMP Degradation by the 12,100 × g Pellet from a Loblolly Pine Callus Homogenate. Experimental Points are Averages of Duplicate Determinations. The Cross-Hatched Area Indicates no Apparent Significant Difference

may be the result of IAA oxidase activity destroying IAA, thus making the IAA treatment similar to the no-auxin treatment after reacting for 10 minutes.

Throughout the first 15 minutes of reaction, degradation products from [8-^{3}H]-cyclic AMP accumulated in solution at much the same rate for all treatments. Within the reaction time interval from 15 to 20 minutes, both [8-^{3}H]-cyclic AMP and total tritiated degradation products increased sharply in the no-auxin and IAA treatments. During the same reaction time interval in the 2,4-D treatment, both [8-^{3}H]-cyclic AMP and total tritiated degradation products continued to decrease and accumulate respectively at about the same rate as in the previous 5-minute interval. Figures 23-25 show the accumulation of individual [8-^{3}H]-cyclic AMP degradation products with time. The increase in total degradation products from 15-20 minutes (Fig. 22) for the IAA and no-auxin treatments were dominated by increases in [8-^{3}H]-adenine and [8-^{3}H]-adenosine, respectively.

These tritiated product increases in the no-auxin and IAA treatment during the 15-20 minute reaction interval resulted from the release of cyclic AMP and its hydrolysis products from a component of the 12,100 × g pellet. The 12,100 × g callus pellet incubated for 15 minutes with [8-^{3}H]-cyclic AMP in the absence of auxin showed a 43% increase in total tritium cpm when treated with TLC II developer (Fig. 26). A similar treatment of the pellet incubated for 15 minutes with [8-^{3}H]-cyclic AMP in the presence of 2,4-D failed to increase the total tritium cpm. Thus, these results indicate that the auxin treatment interfered with the binding of adenine derivatives to a component of the 12,100 × g callus pellet. The [8-^{3}H]-cyclic AMP and [8-^{3}H]-degradation products released in the 15 to 20 minute reaction interval were 38% of the total count present at 15 minutes (assuming the released count was also present but bound to the 12,100 × g pellet at 15 minutes). Most of the bound [8-^{3}H]-adenine derivatives were,

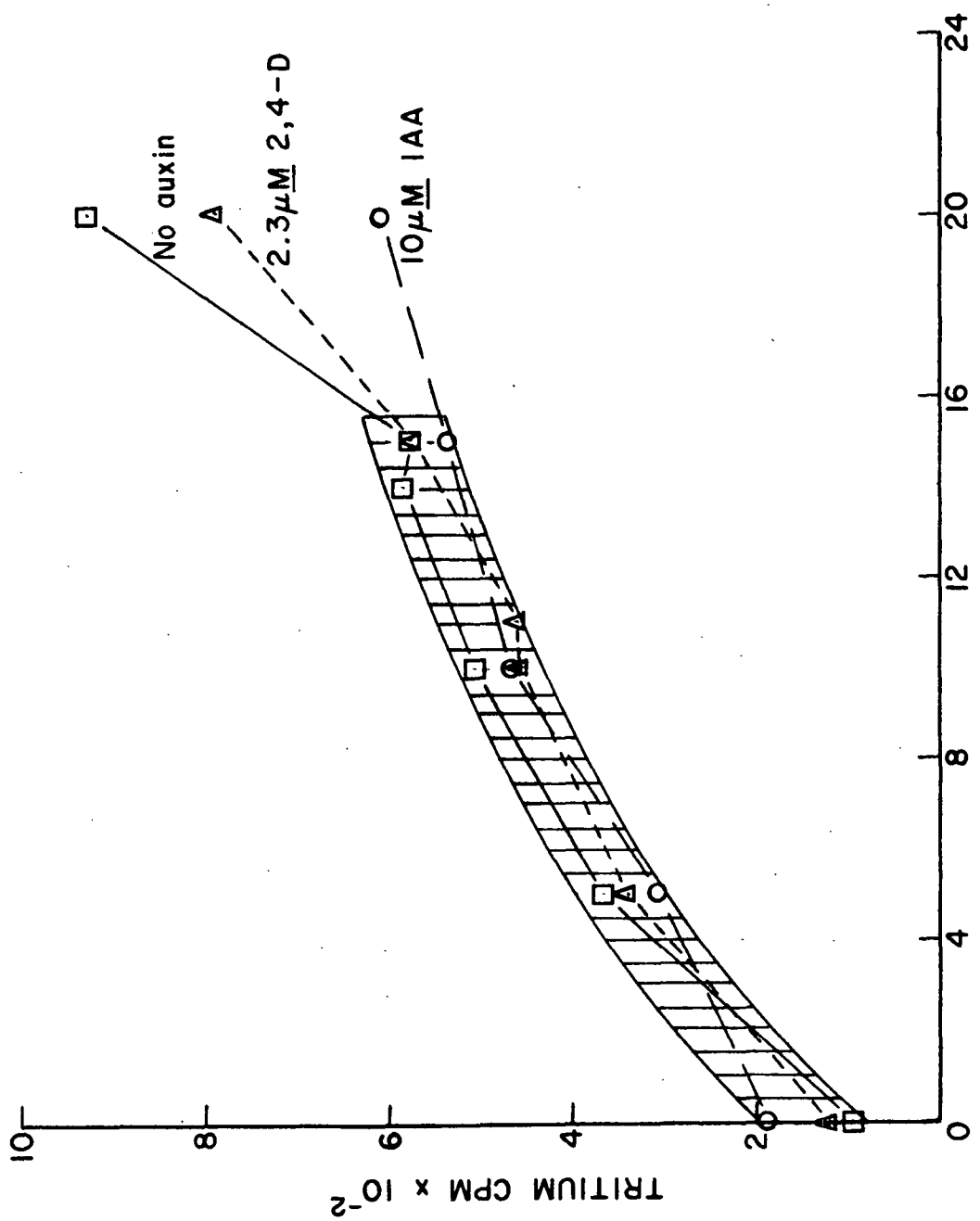


Figure 23. Effect of Auxins on [8-^{3}H]-Adenosine Appearance with Time During [8-^{3}H]-Cyclic AMP Degradation by the 12,100 × g Pellet from a Loblolly Pine Callus Homogenate. Experimental Points are Averages of Duplicate Determinations. The Cross-Hatched Area Indicates no Apparent Significant Difference

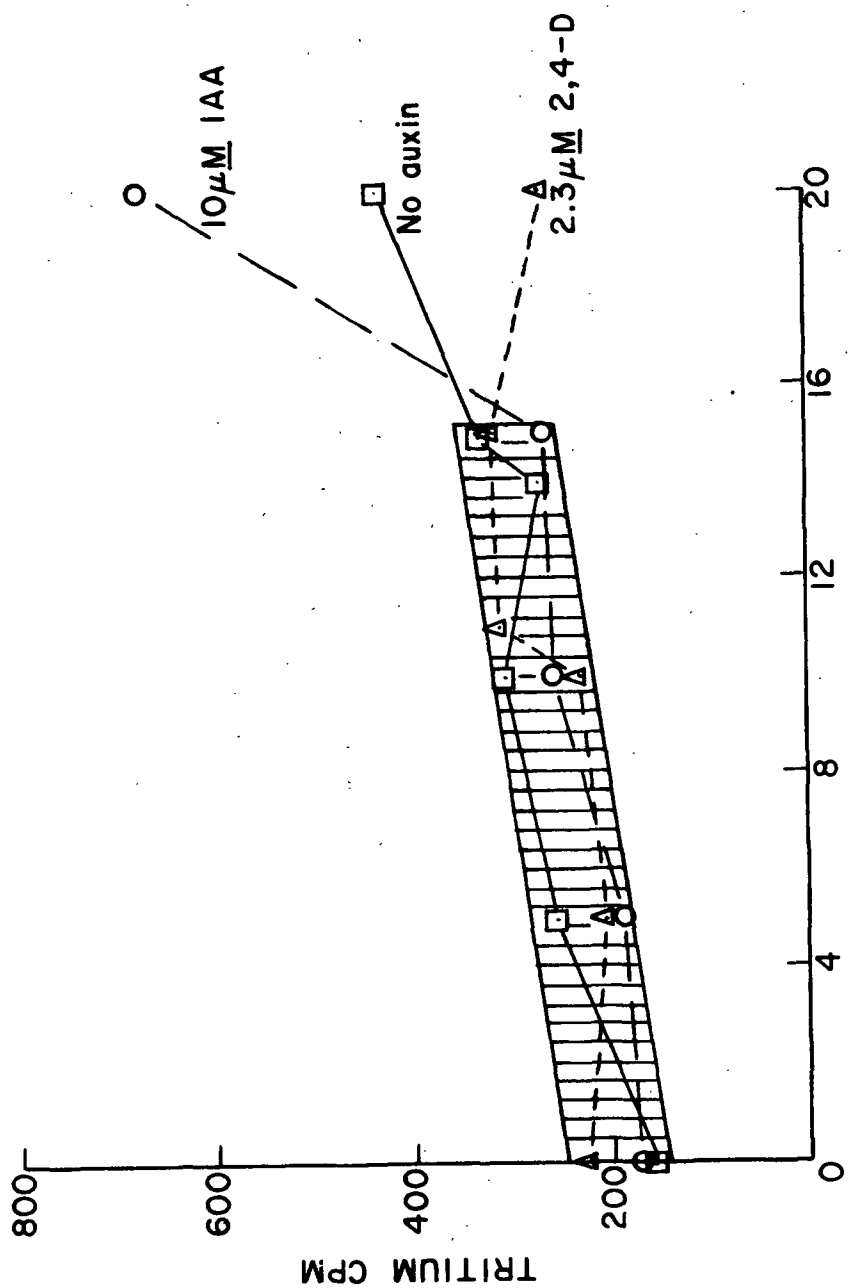


Figure 24. Effect of Auxins on [8-^{3}H]-Adenine Appearance with Time During [8-^{3}H]-Cyclic AMP Degradation by the 12,100 × g Pellet from a Loblolly Pine Callus Homogenate. Experimental Points are Averages of Duplicate Determinations

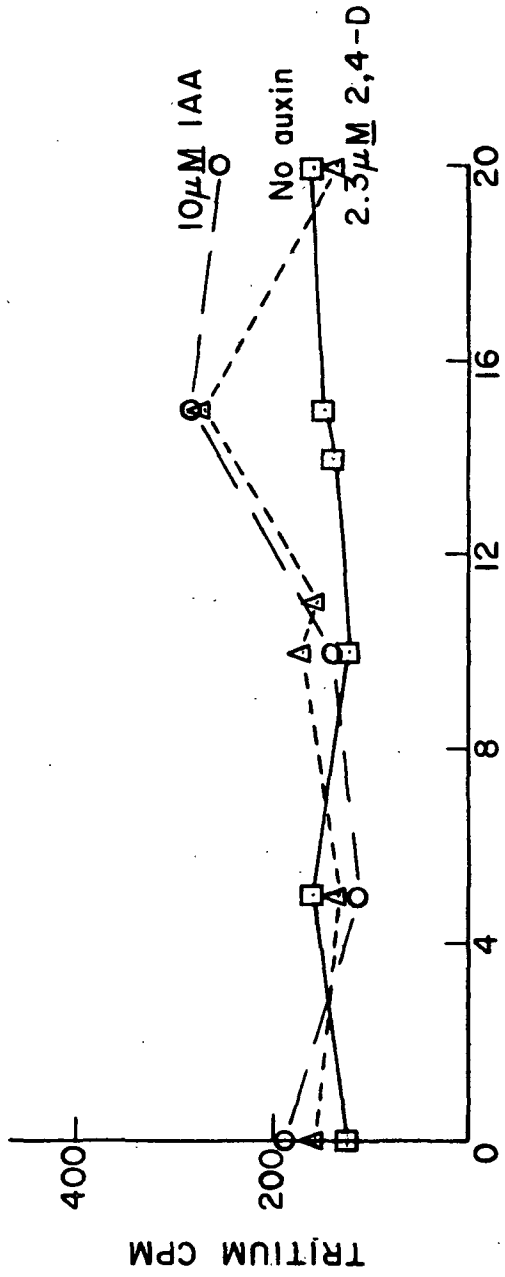


Figure 25. Effect of Auxin on [8-^{3}H]-AMP Appearance with Time During [8-^{3}H]-Cyclic AMP Degradation by the 12,100 × g Pellet from a Loblolly Pine Callus Homogenate. Experimental Points are Averages of Duplicate Determinations

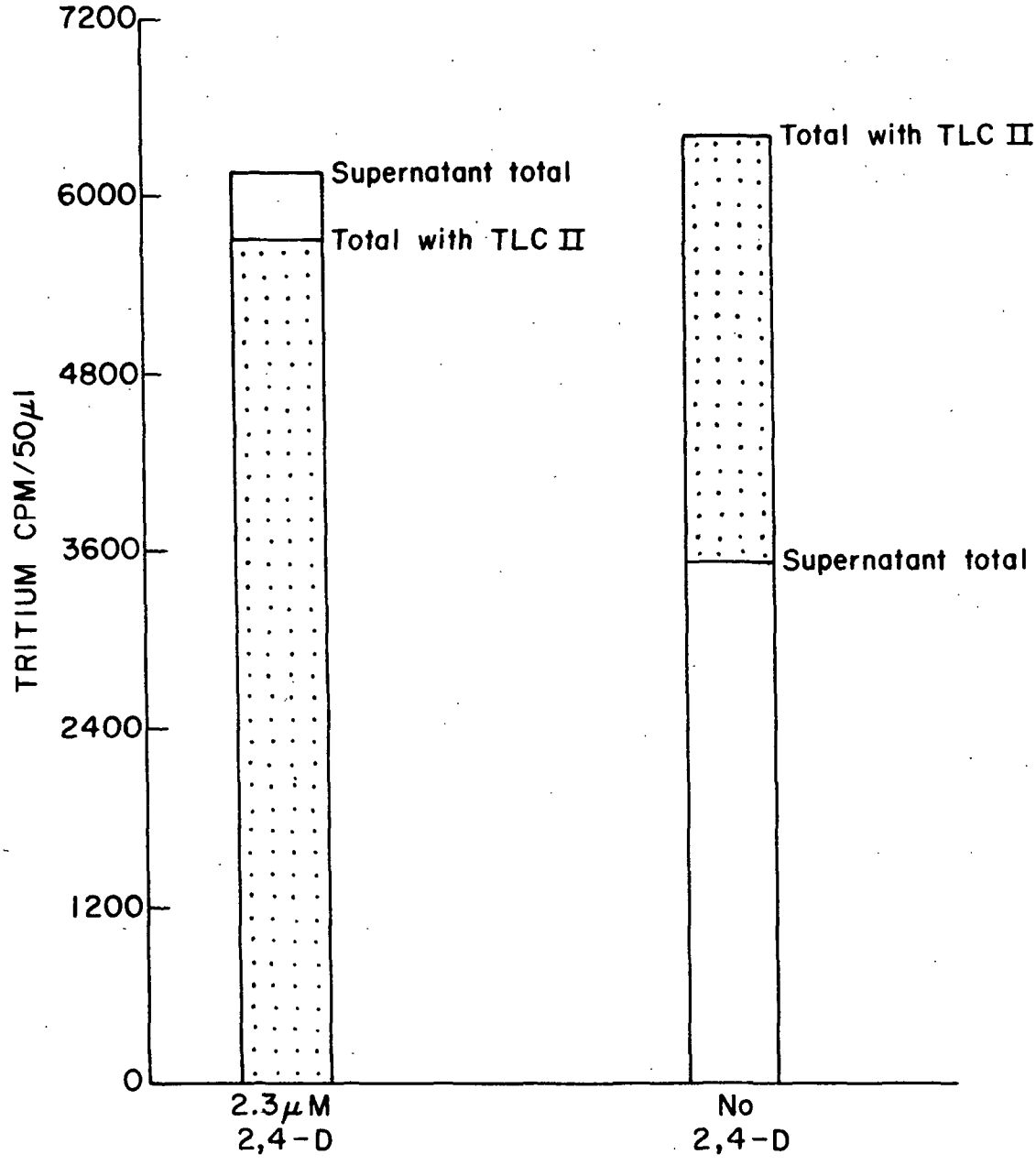


Figure 26. Tritium Activity Recovery After 15-Minute [8-^{3}H]-Cyclic AMP Incubation with the 12,100 × g Pellet from a Loblolly Pine Callus Homogenate and Tritium Activity Released by TLC II Developer

therefore, released during this interval. These results were similar to those obtained when loblolly callus was treated with auxin *in situ*. No influence of auxins on cyclic AMP degradation was observed. Auxin treatment did play a role in the binding of [8-^{3}H]-adenine derivatives to a component of the 12,100 × *g* callus pellet.

AUTORADIOGRAPHIC LOCALIZATION OF [8-^{3}H]-CYCLIC AMP SUPPLIED TO LOBLOLLY PINE CALLUS AND ITS HYDROLYSIS PRODUCTS

As indicated by autoradiography, cyclic AMP was taken up at a much faster rate than cyclic GMP by whole loblolly pine callus cells. This technique also allowed the collective localization of the tritiated cyclic nucleotides and their metabolites in callus cells (Fig. 27). The study of callus whole-cell degradation of cyclic AMP discussed previously showed that cyclic AMP was taken into the cell before degradation began and that at two hours incubation time, about 40% of the [^{3}H]-cyclic AMP supplied had not been hydrolyzed.

Seventy-two percent of the tritium activity centers observed per unit area following incubation of callus with [8-^{3}H]-cyclic AMP were located in the nucleus, 20% in the cytoplasm exclusive of the nucleus, and 8% in vacuoles. Tritium activity centers in the nucleus were observed in the nucleoli as well as in the chromatin. When 2.3-μM 2,4-D was supplied with [^{3}H]-cyclic AMP, the tritium activity center density in the nucleus and cytoplasm was about one-half that when 2,4-D was omitted (Table V). Gordon, *et al.* (60) also found that auxin treatment retarded the uptake of cyclic AMP into maize coleoptiles. The distribution of tritium activity within the loblolly pine callus cells, as observed by light microscopy, was not changed by the presence of auxin.

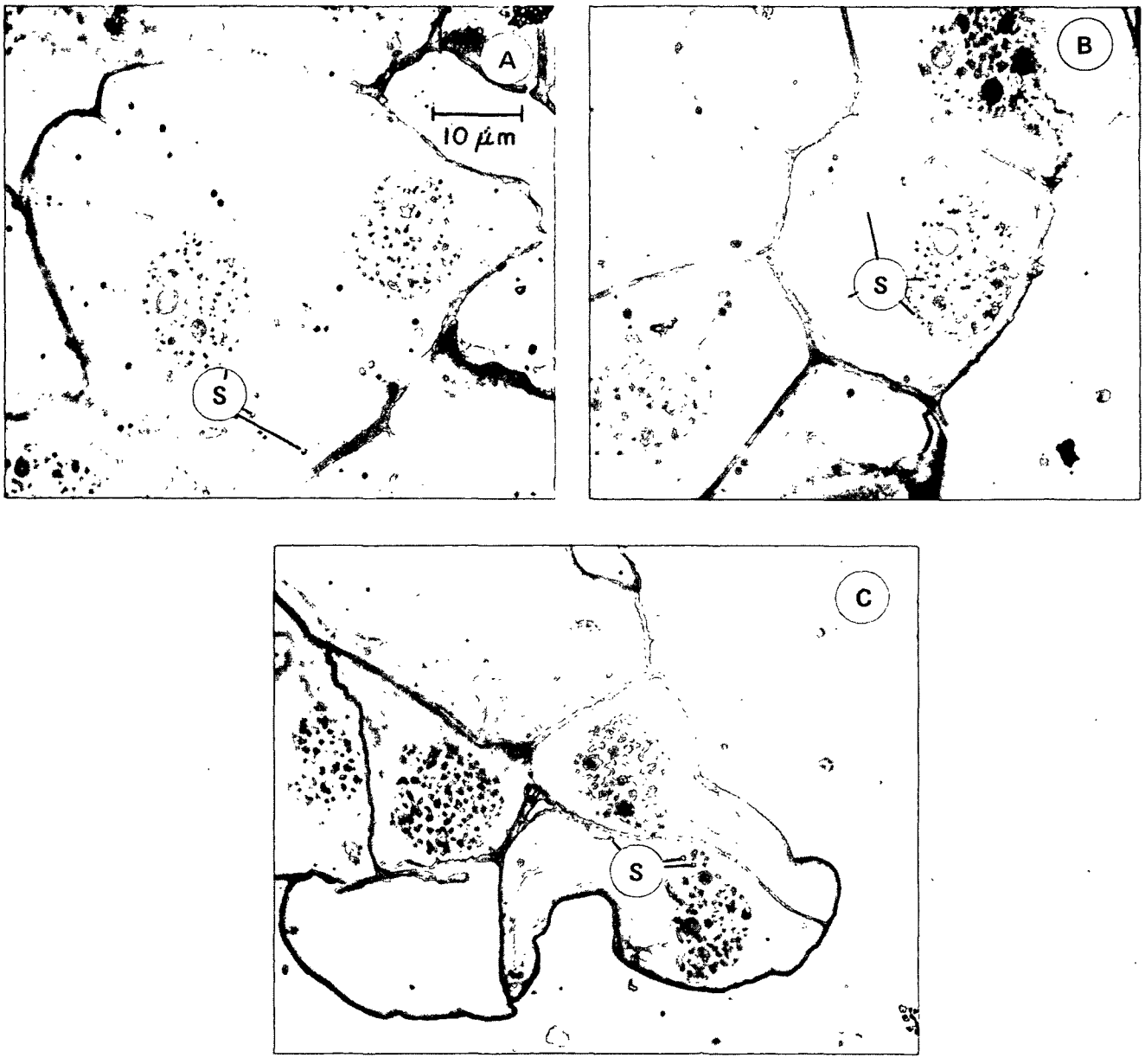


Figure 27. Light Microscope Autoradiographs of Loblolly Pine Callus Sections Following 2-Hour Incubation with [8-^{3}H]-Cyclic AMP or [8-^{3}H]-Cyclic GMP; Fixed with Glutaraldehyde and Osmium Tetroxide-Fixed and Stained with 0.05% Toluidine Blue (pH 6.8). Focus is on the Section and Not the Silver Grains.
A. Callus Incubated with [^{3}H]-Cyclic GMP; B. Callus Incubated with [^{3}H]-Cyclic AMP; C. Callus Incubated with [^{3}H]-Cyclic AMP in the Presence of 2.3-µM 2,4-D; S = Developed Silver Grains, 1400X

TABLE V

AUTORADIOGRAPHIC LOCALIZATION OF TRITIUM ACTIVITY CENTERS PER SQUARE MICROMETER FOLLOWING A TWO-HOUR INCUBATION OF LOBLOLLY PINE CALLUS WITH [8-^{3}H]-CYCLIC AMP[a]

Cellular Component	Treatment	
	No Auxin	2.3-μM 2,4-D
Nucleus	1.70 ± 0.20	0.87 ± 0.09
Vacuole	0.20 ± 0.03	0.14 ± 0.04
Cytoplasm external to nucleus	0.47 ± 0.07	0.23 ± 0.03

[a] Averages ±SEM for 10 cells in 2,4-D treatment and averages for 11 cells in no auxin treatment.

As pointed out earlier, a component of the 600 × g centrifuge pellet fraction from callus selectively bound [8-^{3}H]-cyclic AMP. The selective binding of [8-^{3}H]-adenine derivatives in the nucleus following callus incubation with [8-^{3}H]-cyclic AMP suggests that the component of the 600 × g pellet responsible for [8-^{3}H]-cyclic AMP binding may be the nuclear materials.

CONCLUSIONS

Adenosine 3',5'-cyclic monophosphate was present in unorganized loblolly pine callus grown in the dark on Winton's Medium 10. Cyclic AMP in the callus extracts was bound to a cyclic AMP-dependent protein kinase with the same affinity as authentic (commercially prepared) cyclic AMP. At proper extract dilution, cyclic AMP extracted from the callus was hydrolyzed by a 3',5'-cyclic nucleotide phosphodiesterase at a rate consistent with the hydrolysis of 3',5'-cyclic AMP, but inconsistent with the rate of hydrolysis of cyclic GMP. The only other 3',5'-cyclic nucleotide which might have been present in the partially purified extract was cyclic CMP which has a much lower affinity for the assay protein than cyclic AMP. Furthermore, 3',5'-cyclic NPDE has been reported to be ineffective in catalyzing cyclic CMP hydrolysis (85).

This study showed that the cyclic AMP binding protein assay can be used under the conditions suggested by the Amersham/Searle Corporation to accurately assay cyclic AMP in loblolly pine callus extracts prepared as herein described. During a six-week growth interval following subculture, cyclic AMP levels in callus grown in the dark varied from 24 to 345 pmoles per gram callus wet weight. That callus had cyclic AMP concentration maxima at 2 to 3 and also at 5 weeks from subculture. Cyclic AMP concentrations in the callus were found to increase when the rate of callus wet-weight increase was accelerating. When the rate of callus wet-weight increase decelerated, the cyclic AMP concentration also decreased. The cyclic AMP concentration in the callus was therefore highest during periods of rapid callus growth.

Adenyl cyclase activity was detected in the 600 × g supernatant of a loblolly pine callus homogenate when assayed in the presence of sodium fluoride. Since sodium fluoride was found to strongly inhibit cyclic AMP hydrolysis by a

callus homogenate, it appears that cyclic AMP degradation reactions dominate when the callus cellular organization is physically disrupted. ATP levels present in loblolly pine callus homogenates were apparently not sufficiently high to saturate the adenyl cyclase catalytic sites, revealed by the fact that cyclic AMP accumulation was stimulated when ATP was supplied in the assay medium.

Adenosine is the most rapidly accumulated compound resulting from cyclic AMP degradation by loblolly pine callus whole cells or broken cell preparations. 3'-AMP and 5'-AMP are also present at a low and constant level. Adenine increased at a very slow rate during cyclic AMP degradation. The relatively high nucleotidase activity observed in the 12,100 × g pellet accounts for the low and constant AMP levels present compared to the higher adenosine levels which occur during cyclic AMP degradation. Both [8-^{14}C]-5'-AMP and [8-^{14}C]-adenosine were converted to [8-^{14}C]-adenine by the callus. Other adenine derivatives that were present might also have been degraded to adenine, but this possibility was not investigated.

Most of the cyclic AMP degradative activity was concentrated in the 12,100 × g pellet fraction from a callus homogenate. The remaining portion of the cyclic AMP degradative activity results from the catalytic activity of solubilized enzymes. Brewin and Northcote (38) similarly found that the majority of the cyclic NPDE activity from soybean callus homogenates was found in a 12,000 × g pellet. Up to 35% of the activity from their preparations could be sedimented at less than 1000 × g and appeared to be associated with the plasmalemma. A lag period of at least 15 minutes in cyclic AMP degradation by whole loblolly pine callus cell preparations further supports the conclusion that cyclic AMP enters the cytoplasm of the callus cell before its degradation begins.

A secondary objective of this thesis was to determine the effect, if any, of auxins on cyclic AMP levels in the callus. Light-microscope autoradiographic studies showed that tritiated activity centers resulting from incubating whole callus cells with [8-^{3}H]-cyclic AMP were concentrated in the nuclei of the callus cells. The presence of 2.3-μM 2,4-D had no effect on the distribution of these activity centers between the nucleus and the cytoplasm. When 2,4-D was present during the incubation, the uptake of [8-^{3}H]-cyclic AMP into callus cells was about one-half that observed in the absence of 2,4-D, as estimated by the tritiated activity center density. Therefore, 2,4-D appears to play a role in the callus' plasmalemma permeability to cyclic AMP. Under the same incubation conditions, [8-^{3}H]-cyclic GMP is taken into the callus at a much slower rate than is cyclic AMP.

In addition, 2,4-D was found to interfere with a reduction in the amount of cyclic AMP extractable from the callus with time. The duration of this effect, however, was less than one hour following treatment. Auxins were found to have no effect on the rate of cyclic AMP degradation in the callus. They did, however, play a role in the binding of [8-^{3}H]-adenine derivatives to some component of the 12,100 × g pellet. The control of this binding appears to be complex and may also involve other substances occurring naturally in the callus.

This work is the first reported study of either the presence or the metabolism of cyclic AMP in tissue or cultured cells from a coniferous tree species. Although numerous published reports of the presence of cyclic AMP in higher plants have been justifiably criticized for the methods used to validate the assay response, in this work the assay response was investigated extensively and the results consistently indicated a valid assay for cyclic AMP. The finding that the endogenous cyclic AMP concentrations in the aseptic callus cultures fluctuated

in a direct relationship with the growth rate is the first report of changes in cyclic AMP levels in higher plants during an extended growth period. Others have reported exogenously supplied cyclic AMP to stimulate the cellular growth rate in the presence of auxin. Utilizing a valid assay method for cyclic AMP, adenyl cyclase activity was detected for the first time in an unorganized aggregation of cells from a higher plant. Since adenyl cyclase activity has now been verified in the unorganized callus, it appears feasible that cyclic AMP could play a role in cellular differentiation and the tissue organization process in loblolly pine.

SUGGESTIONS FOR FUTURE RESEARCH

This research has established the presence of cyclic AMP and enzymes capable of controlling its concentration in unorganized loblolly pine callus. Substantial additional research will be needed to bring to light the significance of cyclic AMP in the growth and development of the callus. A few fruitful areas of further research suggested by this work are presented in the following paragraphs.

Tritiated activity centers located in callus nuclei resulting from [8-^{3}H]-cyclic AMP supplied to the callus were observed in this work, but not identified. The elucidation of tritiated compounds present and the nuclear components with which they are associated could lead to the significance, if any, of the concentration of tritiated activity in callus nuclei following its incubation with [8-^{3}H]-cyclic AMP.

A similar study of the tritiated activity center distribution in the cytoplasm following callus incubation with [8-^{3}H]-cyclic AMP could reveal either organelles involved in cyclic AMP metabolism or the cellular sites of its action. The unknown component(s) of the 12,100 × g centrifuge pellet shown to bind [8-^{3}H]-adenine derivatives might be identified by electron-microscope autoradiography techniques using postdevelopment staining. Plant hormone effects might also be observed in such a study.

Loblolly pine callus grown in the dark was found to have fluctuating cyclic AMP levels during a growth passage of 6 weeks. The study of exogenous applications of plant hormones as well as sugars available to the callus and their effects on cyclic AMP levels in the callus might be undertaken to investigate the causes of these cyclic AMP fluctuations.

A direct relationship was observed between cyclic AMP concentrations and the rate of callus wet-weight increase, but the cause and effect aspect of this condition was not investigated. The study of this relationship could possibly lead to an understanding of initial cellular responses to (a) increased cyclic AMP concentrations or to (b) callus growth substances which stimulate the cyclic AMP increases.

GLOSSARY AND SPECIAL ABBREVIATIONS

ADP - 5'-Adenosine diphosphate

AMP - Refers collectively to adenosine monophosphate

3'-AMP - 3'-Adenosine monophosphate

5'-AMP - 5'-Adenosine monophosphate

α-Amylase - An enzyme which catalyzes hydrolytic cleavage of α-(1→4) glucose linkages

ATP - 5'-Adenosine triphosphate

α-[^{32}P]-ATP - ATP having ^{32}P in the phosphate group closest to adenosine

γ-[^{32}P]-ATP - ATP having ^{32}P in the terminal phosphate group

Auxins - Plant growth hormones of several types which cause cell enlargement, apical dominance, and root initiation; a natural auxin is 3-indoleacetic acid

Callus culture - Proliferation from a parental explant of many cells in protoplasmic continuity, but having no equivalence with any normal tissue

Chromatin - A nucleoprotein forming part of chromosomes; made up of protein, DNA, and RNA

Cyclic AMP or 3',5'-cyclic AMP - Adenosine 3',5'-cyclic monophosphate

2',3'-Cyclic AMP - Adenosine 2',3'-cyclic monophosphate

Cyclic CMP - Cytidine 3',5'-cyclic monophosphate

Cyclic GMP - Guanosine 3',5'-cyclic monophosphate

Cyclic NPDE - Cyclic nucleotide phosphodiesterase, an enzyme which catalyzes the hydrolysis of phosphate ester bonds in cyclic nucleotides resulting in the formation of acyclic nucleotides

Cyclic nucleotide - A nucleotide in which the phosphate group forms a cyclic diester with its sugar unit

Cytokinin - A class of plant growth hormones associated with cell division; all have the N^6-substituted aminopurine unit in their structure

Cytoplasm - The protoplasm of a cell excluding the nucleus and other organelles

2,4-D - 2,4-Dichlorophenoxyacetic acid; a synthetic auxin

DNA - Deoxyribonucleic acid, polyneucleotide having specific sequence of deoxyribonucleotide units and serving as the carrier of genetic information in chromosomes

EDTA - Disodium ethylenedinitrilotetraacetate

GA_3 - Gibberellic acid

Gibberellin - A class of plant growth hormones which stimulate height growth of dwarf plants and/or induce α-amylase synthesis; all contain the gibbane unit in their structure

Glycogen - A highly branched polymer of glucose found in animals having α-(1→4) glycosidic linkages in the linear chain with branching occurring at C-6

Glycogenolysis - The enzymatically catalyzed hydrolysis of glycogen resulting in the liberation of glucose units

IAA - 3-Indoleacetic acid, a naturally occurring auxin

IAA-oxidase - An enzyme which catalyzes the oxidation of 3-indoleacetic acid to 3-methylene-oxindole

Inhibitor - A substance which limits or destroys the catalytic activity of an enzyme

mRNA - Messenger RNA

Nucleolus - A small dense body rich in RNA and protein found in the nucleus of the cell

Nucleotide - A compound consisting of a purine or pyrimidine base covalently linked to a pentose which is phosphorylated at one of its hydroxyl groups

Passage - The duration of growth of callus from one subculture to another

Plasmalemma or plasma membrane - Unit membrane surrounding the cell cytoplasm

pmole - Picomole, 1×10^{-12} mole

Protein kinase - An enzyme which catalyzes the transfer of a phosphate group from ATP to a protein

RNA - Ribonucleic acid, a polyribonucleotide linked by successive 3',5'-phosphodiester linkages

Subculture - Cutting solid callus into small cubes for transfer to fresh medium

<u>tris</u> - <u>tris</u>-(Hydroxymethyl)aminomethane

Unorganized callus - Callus which may or may not contain one or more isolated cells which are differentiated

Vacuole - The usually large, centrally located portion of the plant cell limited by a unit membrane and filled with dilute salt and sugar solution

LIST OF SCIENTIFIC AND COMMON NAMES

Barley	(*Hordeum vulgare*)
Bengal gram	(*Cicer arietinum*)
Broad bean	(*Vinca faba*)
Carrot	(*Daucus carota*)
Chinese cabbage	(*Sinapis pekinensis*)
Coleus	(*Coleus rhenaltianus*)
Douglas-fir	(*Pseudotsuga menziesii*)
Duckweed	(*Lemna gibba* G3)
Hazel	(*Corylus avellana*)
Honey locust	(*Robinia pseudoacacia*)
Jerusalem artichoke	(*Helianthus truberosus*)
Kelp	(*Laminaria saccharina*)
Kidney bean	(*Phaseolus vulgaris*)
Lettuce	(*Lactuca satvia*)
Loblolly pine	(*Pinus taeda*)
Madagascar periwinkle	(*Vinca rosea*)
Maize (corn)	(*Zea mays*)
Mustard	(*Sinapis alba*)
Oat	(*Avena satvia*)
Pea	(*Pisum satvia*)
Potato	(*Solanum tuberosum*)
Quaking aspen	(*Populus tremuloides*)
Soybean	(*Dolichos soja*)
Sycamore maple	(*Acer pseudoplatanus*)
Tobacco	(*Nicotiana tabacum*)
Tomato	(*Lycopersicon esculentum*)

Bacterium	(*Brevibacterium liquefaciens*)
Bacterium	(*Escherichia coli*)
Fresh water alga	(*Chlamydomonas reinhardtii*)
Mold	(*Neurospora crassa*)
Slime mold	(*Dictyostelium discoideum*)
Yeast	(*Sacchromyces fragilis*)

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APPENDIX I

CALLUS GROWTH MEDIA

Calluses were grown on Winton's Medium 10 the composition of which is listed in Table VI, and on a modification of Medium 10, as shown below.

TABLE VI

COMPOSITION OF WINTON'S MEDIUM 10

Inorganic Nutrients	Mg/Liter	Organic Supplements	Mg/Liter
NH_4NO_3	1650	Nicotinic acid	0.5
KNO_3	1900	Pyridoxine • HCl	0.1
$CaCl_2 \cdot 2H_2O$	440	Thiamine • HCl	0.1
$MgSO_4 \cdot 7H_2O$	370	β-Naphthoxyacetic acid	5.0
KH_2PO_4	170	N^6-Benzylaminopurine	0.1
H_3BO_3	6.2	Inositol	100
$MnSO_4 \cdot 4H_2O$	16.9	Asparagine	100
$ZnSO_4 \cdot H_2O$	10.6	Sucrose (3%)	30,000
KI	0.83	Bacto agar (1%)	10,000
$Na_2MoO_4 \cdot 2H_2O$	0.25	pH[b]	5.6 ± 0.2
$CuSO_4 \cdot 5H_2O$	0.025		
$CoCl_2 \cdot 6H_2O$	0.025		
Fe (as FeEDTA)[a]	5.6		

[a] 5 ml/l of a stock solution containing 5.57 g $FeSO_4 \cdot 7H_2O$ and 7.45 g Na_2-EDTA per liter of H_2O.

[b] Adjusted with 1% NH_4OH or 1N HCl before the addition of agar and autoclaving.

WINTON'S IPA-2 MEDIUM

This agar medium is the same as Winton's Medium 10 (shown in Table VI) in all respects except for the following changes in concentration and additions:

Concentration Changes:	Mg/Liter
Thiamine • HCl	3.0
KH_2PO_4	340
Constituents Added:	
Ascorbic acid	1.0
Adenine sulfate	100
N^6-(Δ^2-Isopentenylamino)purine*	0.01
Folic acid	0.1
Riboflavin	0.1
Biotin	0.1

*Substituted for N^6-benzylaminopurine which was present in Medium 10.

APPENDIX II

CYCLIC AMP ISOLATION FOR ASSAY

NUCLEOTIDE EXTRACTION

Nucleotides were extracted from the callus and protein precipitated by the following method:

1. Callus to be extracted was removed from the agar medium and quickly wrapped in a cheesecloth sack tied at the top with copper wire. The sack containing the callus sample was then immersed in liquid nitrogen to stabilize the endogenous cyclic AMP level.

2. The frozen callus mass was then removed from the cheesecloth and powdered using stainless steel beaker and pestle, both precooled with liquid nitrogen.

3. The powdered callus was transferred to a cold centrifuge tube and mixed with ice-cold 6% TCA (approximately 5 ml/g callus powder). 3000-4000 Cpm of [8-^{3}H]-cyclic AMP (specific activity 21.8 Ci/mmole purchased from International Chemical and Nuclear Corporation) was added to the TCA slurry in a 50-µl volume to estimate cyclic AMP recovery.

4. The TCA callus powder mixture was then stirred and placed in the deep freeze at -20°C until all samples to be assayed had been processed through Step 4.

5. The frozen TCA callus powder mixtures were thawed and mixed thoroughly, then the solids sedimented at 21,600 x g for 15 minutes at 2°C.

6. The supernatants were extracted five times with two volumes (each time) of water-saturated ethyl ether to remove TCA. The sedimented solids were freeze-dried for dry weight determinations.

7. The 50-ml Erlenmeyer flasks containing the water layer were heated 3 minutes in a water bath to drive off residual ether.

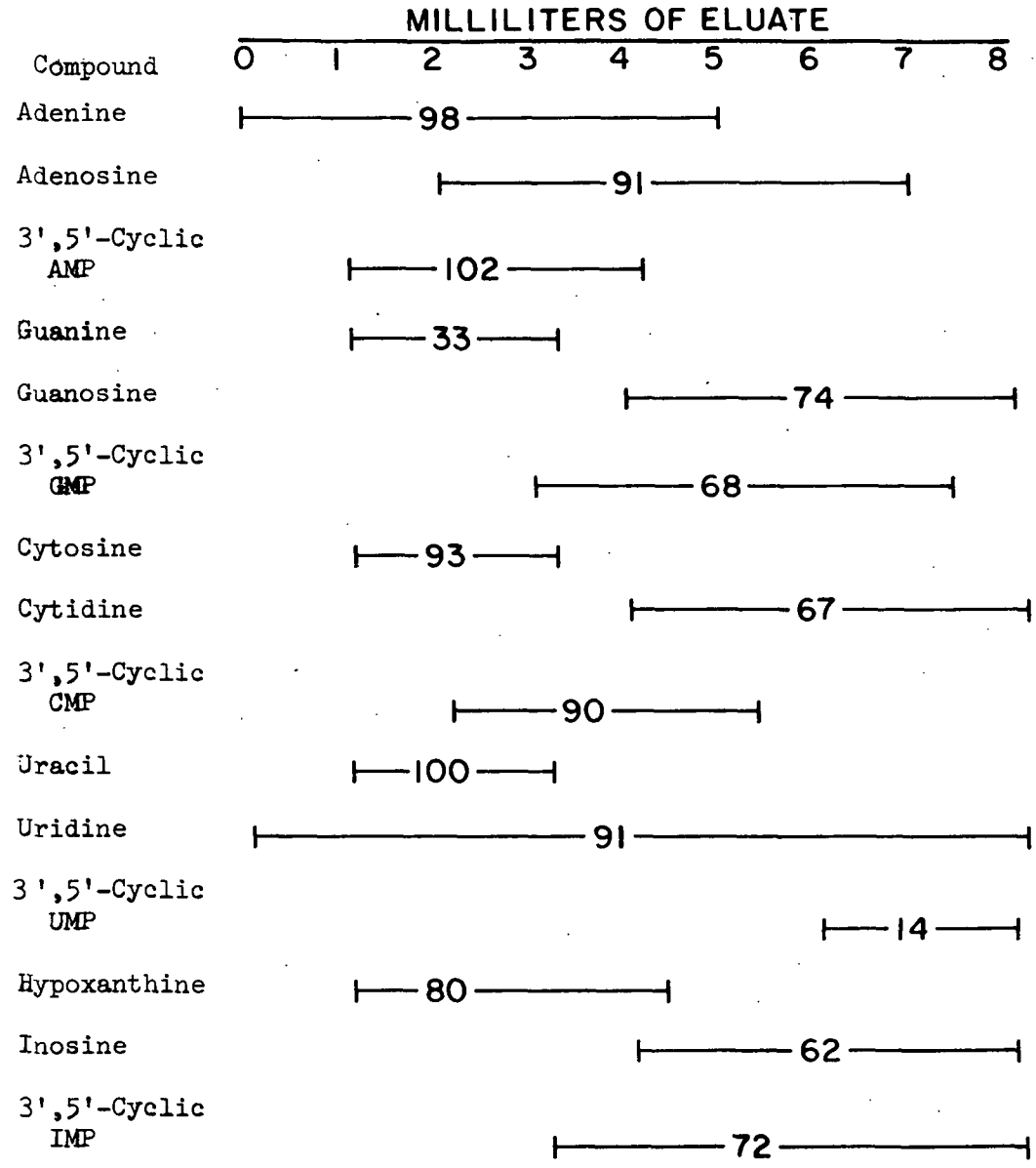
8. The extracts were then lyophilized to concentrate for further purification.

All samples to be assayed were processed through Step 8 as quickly as possible to minimize acid-catalyzed hydrolysis of cyclic AMP.

Nucleotide extracts were chromatographed on alumina (76); typical results are listed in Table VII. The recovery of [8-^{3}H]-cyclic AMP from a typical chromatogram is listed in Fig. 28.

TABLE VII

NUCLEOTIDE, NUCLEOSIDE, AND NITROGENOUS BASE ELUTION FROM ALUMINA COLUMNS, PERCENT OF TOTAL LOAD RECOVERED (76)[a]



[a] Zero percent of the following compounds was recovered in the first 8 ml: 2',3'-cyclic AMP, AMP, 5'-ADP, 5'-ATP, GMP, 5'-GDP, 5'-GTP, 2',3'-cyclic CMP, CMP, 5'-CDP, 5'-CTP, 2',3'-cyclic UMP, UMP, UTP, IMP, ITP.

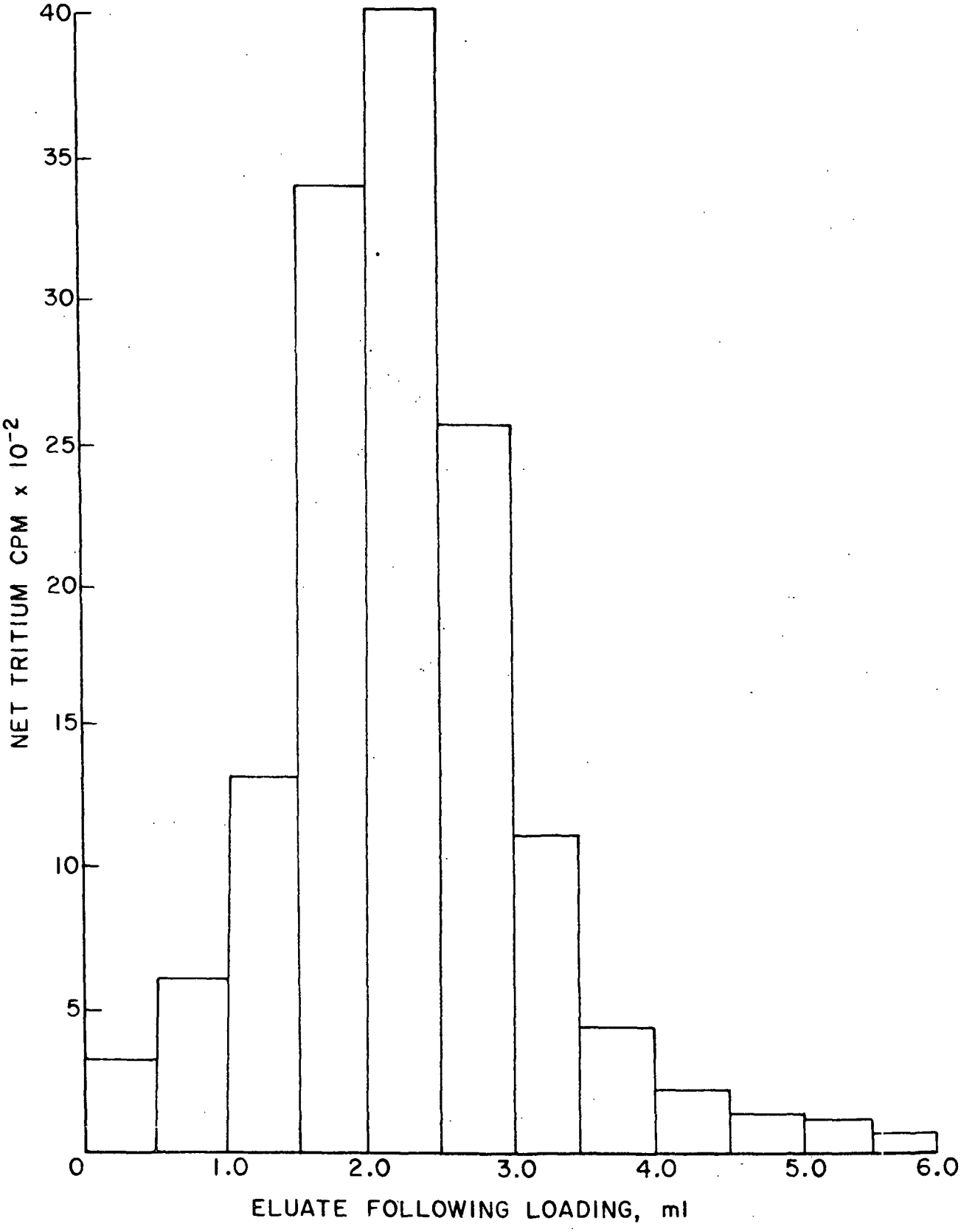


Figure 28. [8-^{3}H]-Cyclic AMP Elution from Alumina with 8.3 mM tris-HCl (pH 7.5)

APPENDIX III

CALCULATION OF CYCLIC AMP CONCENTRATION FROM BINDING-PROTEIN ASSAY DATA

The concentrations of cyclic AMP were calculated from protein-binding data, as shown in Table VIII, and the standard curve (Fig. 29).

TABLE VIII

BINDING-PROTEIN ASSAY DATA

Assay Sample	[^{3}H] Cpm of Assay Supernatant[a]	Net [^{3}H] Cpm of Assay Supernatant[a]	C_o/C_x	Pmoles Cyclic AMP[b] per 50 µl
Callus extract	1684	1571		
Callus extract	1721	1608		
Average		1589	2.05	1.4
Assay buffer	3388	3275		
Assay buffer	3357	3244		
Average		3261	1.00	
Assay buffer without binding protein	119			
	107			
Average	113			

[a] 100 µl aliquots were counted.

[b] Value determined from the standard curve.

CALCULATION OF CYCLIC AMP CONCENTRATION

Dry weight of callus extracted = 0.0722 g

[^{3}H]-Cyclic AMP recovered in extraction = 37.5%

Fraction of total extract assayed = 0.075

Cyclic AMP recovery factor = (0.375)(0.075) = 0.0253

$$\frac{\text{pmoles cyclic AMP}}{\text{10 mg dry weight}} = \frac{\text{1.4 pmoles cyclic AMP/50 µl}}{(7.22 \times 10^{-2}\ \text{g dry wt.})(0.0253)} = 7.6\ \text{pmoles cyclic AMP/}$$

1×10^{-2} g callus dry weight

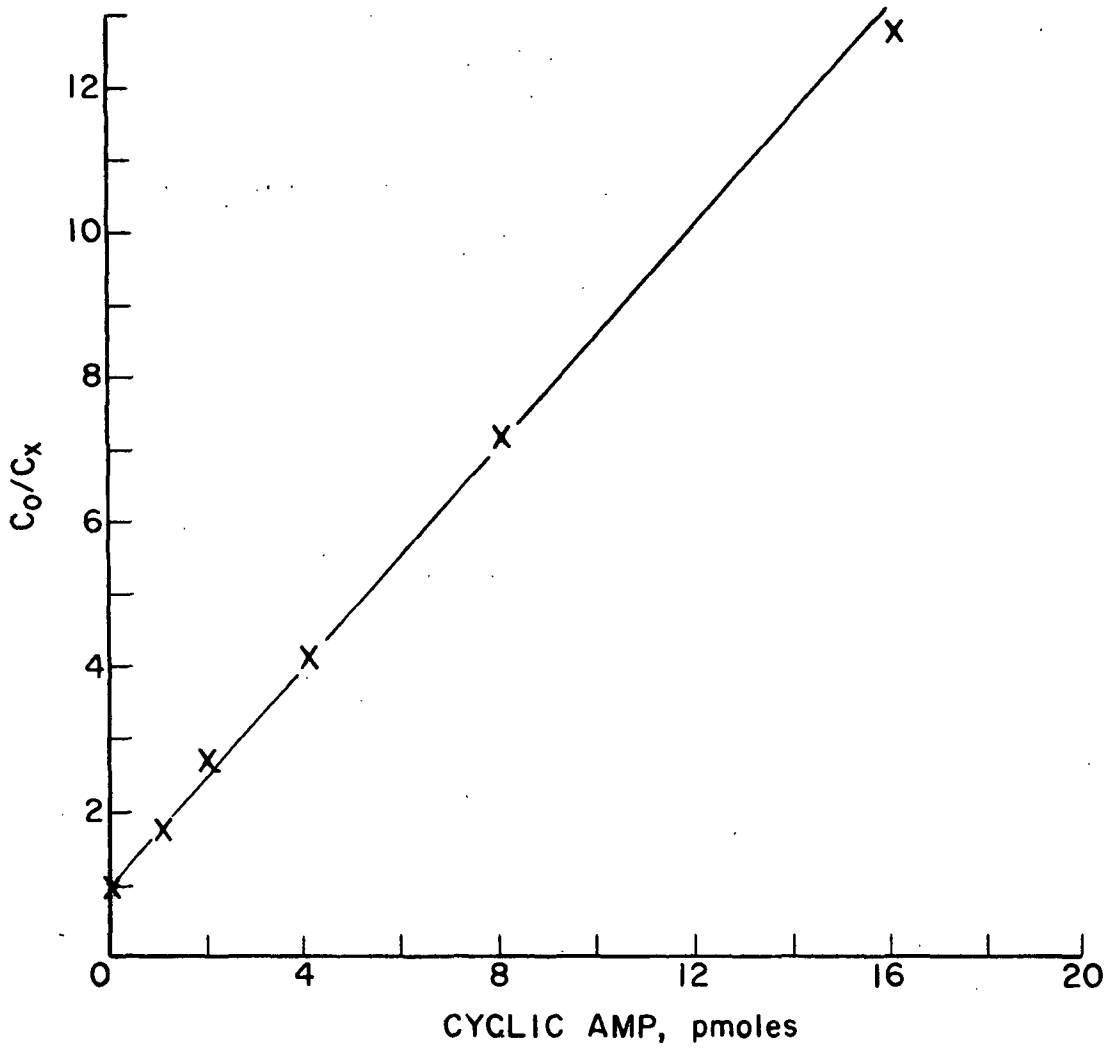


Figure 29. Cyclic AMP Assay Standard Curve. C_o = Tritium CPM in Presence of no Unlabeled Cyclic AMP; C_x = Tritium CPM in Presence of x pmoles Unlabeled Cyclic AMP

APPENDIX IV

ENZYMATIC ACTIVITIES IN THE COMMERCIAL 3',5'-CYCLIC NUCLEOTIDE PHOSPHODIESTERASE PREPARATION AS REPORTED BY SIGMA CHEMICAL COMPANY

Table IX shows several active enzymes were present in the 3',5'-cyclic nucleotide phosphodiesterase preparation obtained from the Sigma Chemical Co.

TABLE IX

ENZYME ACTIVITIES ACCOMPANYING 3',5'-CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

Enzyme	Substrate	Activity[a] Units × 10^3/mg Protein, Lot No. 120C-7740	Activity[a] Units × 10^3/mg Protein, Lot No. 120C-7751-9	pH
Phosphodiesterase, 3',5'-cyclic nucleotide	3',5'-Cyclic AMP	450	200	7.5
5'-Nucleotidase	5'-AMP	1.35	0.97	7.5
5'-ATPase	5'-ATP	2.8	1.1	7.5
Pyrophosphatase, inorganic	Pyrophosphate (Na)	14.6	7.7	7.2
Phosphatase, alkaline	p-Nitrophenyl phosphate	0.042	0.055	10.4
Phosphodiesterase, 2',3'-cyclic nucleotide	2',3'-Cyclic AMP	2.5	2.6	7.5

[a]All reactions were at 30°C. One activity unit catalyzes the conversion of 1.0 μmole of substrate to products per minute at the pH indicated.

APPENDIX V

DECREASE OF TRITIUM COUNTING RATE OF [8-^{3}H]-CYCLIC AMP DURING SCINTILLATION COUNTING

The counting rate of tritium in [8-^{3}H]-cyclic AMP, shown in Table X, decreased with time of contact with the scintillation cocktail.

TABLE X

DECREASE IN [8-^{3}H]-CYCLIC AMP COUNTING RATE WITH TIME IN THE PRESENCE OF MACHERY-NAGEL NM POLYGRAM $CEL_{300}UV_{254}$ TLC COATING

Time After Cocktail[a,b] Addition, min	Tritium Activity, cpm; counting error %[c]	External Standard Number
Sample 1:		
10	8433	6.9
20	8266	7.0
30	8228	6.9
40	8176	6.9
50	8122	7.0
60	8042	6.9
70	8109	6.9
80	8061	6.9
90	7949	6.9
Sample 2:		
10	3623 ± 1.5	6.7
300	2298 ± 1.5	6.8
2880	1768 ± 2.0	6.8

[a] Time at the end of a 10-minute counting interval.

[b] The liquid scintillation counting cocktail was Beckmann Cocktail D.

[c] Counting error was ±1.0% unless otherwise indicated.

APPENDIX VI

ASSAY OF CYCLIC AMP IN LOBLOLLY PINE CALLUS WITH AND WITHOUT CALLUS EXPOSURE TO PHOTOGRAPHIC LIGHTING

The influence of photographic lighting on the cyclic AMP content of loblolly pine callus is shown in Table XI.

TABLE XI

ASSAY OF CYCLIC AMP IN LOBLOLLY PINE CALLUS

Loblolly Pine Callus Samples		Pmoles Cyclic AMP/10 Mg Dry Wt.
Callus photographed:		
	1	11.7
	2	11.4
	3	26.0
	4	17.4
	Average	16.6
Callus not photographed:		
	1	7.6
	2	17.6
	Average	12.6

APPENDIX VII

SUBSTANCES REPORTED BY AMERSHAM/SEARLE CORPORATION TO HAVE NO SIGNIFICANT INFLUENCE ON THEIR CYCLIC AMP BINDING PROTEIN ASSAY

Compound	Maximum Concentration
5'-Adenosine triphosphate	5 mM
Theophylline	32 mM
Potassium fluoride	80 mM
Magnesium ions	10 mM
Calcium ions	80 mM
EDTA	40 mM
Sodium chloride	2 mM
tris-HCl assay buffer	0.5M

APPENDIX VIII

HIGH TEMPERATURE BIO-GEL P-2 CHROMATOGRAPHY

BIO-GEL P-2 COLUMN PREPARATION

Before the column was assembled, the sections were coated twice using a 1% dimethyldichlorosilane solution as described by Bio-Rad Laboratories (89). The assembled column, shown in Fig. 30, was then packed with Bio-Gel P-2 (<400 mesh) polyacrylamid gel (Bio-Rad Laboratories) as follows:

1. The gel (200 grams) was added slowly, with continuous stirring, to 1800 ml of 0.25M citrate buffer, pH 4.4. When addition was complete, the slurry was aspirated overnight while being stirred from below with a magnetic stirrer.

2. The column with a 15-cm extender attached, was filled with degassed citrate buffer and a reservoir containing the degassed gel slurry was attached to the top of the extender. The gel slurry was stirred slowly using an overhead stirrer as the gel settled into the column.

3. After a 10-cm gel bed had formed, the stopcock at the bottom of the column was opened to aid in packing. Buffer collected at the bottom of the column was periodically added back to the reservoir.

4. After the gel bed had reached the top of the column extender, the reservoir was replaced by a teflon plug, fitted with 3-mm outside diameter teflon tubing, which was cemented with Epoxy 907 adhesive to the end of the extender.

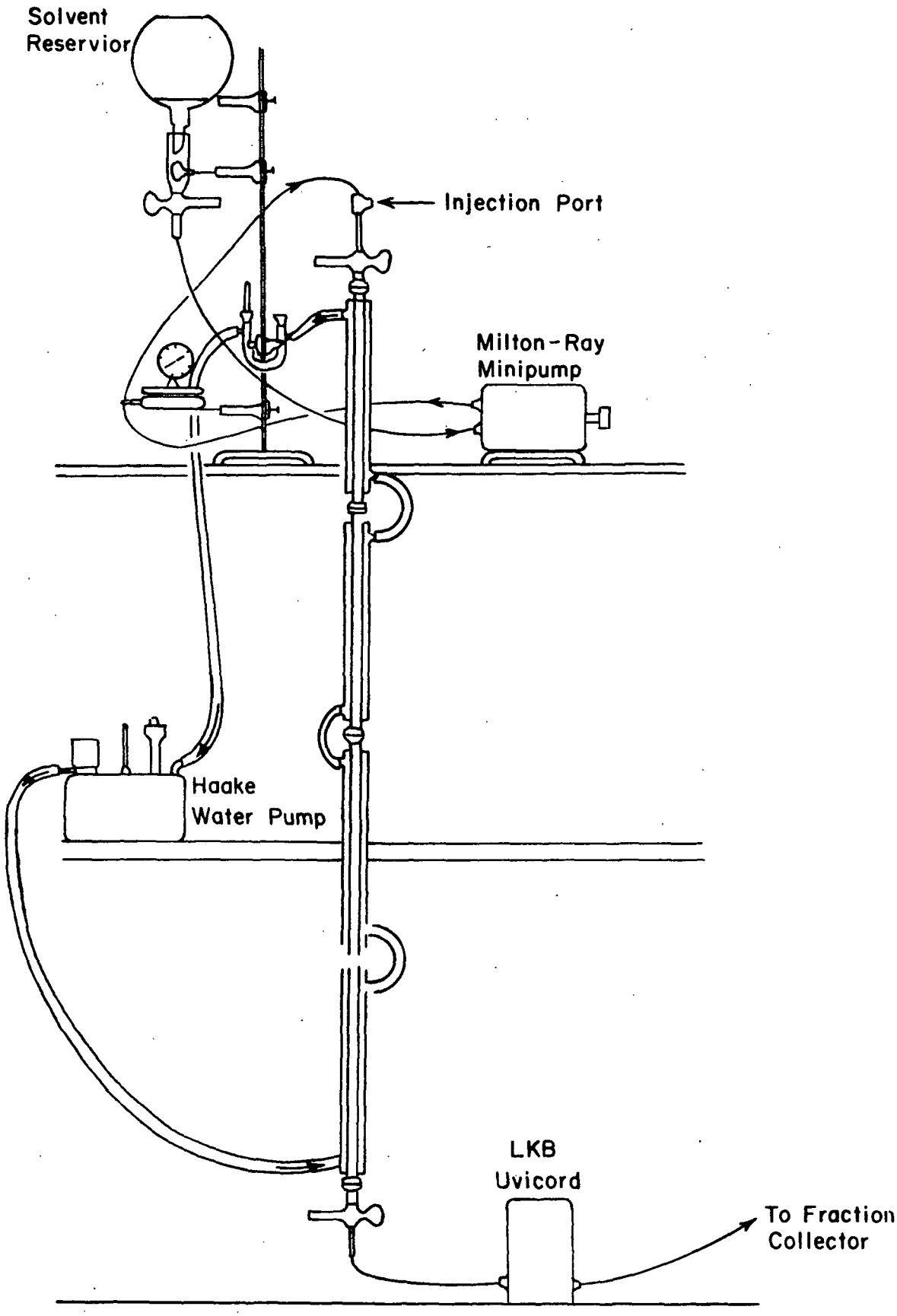


Figure 30. Bio-Gel P-2 Column and Accessories

5. Citrate buffer (0.25M, pH 4.4) containing 0.5 ml Bri-J-35 (polyoxyethylene lauryl ether; formula AR 110-62, Technicon) per liter, was pumped through the gel at the rate of 50 ml per hour for 24 hours using a Milton Roy Minipump (Milton Roy Co.). The Bri-J-35 is added to aid in gel dispersion for more uniform packing.

6. Additional gel was added to the extender and degassed citrate buffer without Bri-J-35 was pumped through the column for 10 hours.

7. The column extender was replaced by the top stopcock fitting which was then filled with degassed buffer and the column was ready for operation.

Column loading was at ambient temperature even though the column was maintained at 69-74°C during chromatography. Under the conditions described, the elution volumes for several nucleotides and related compounds are listed in Table XII.

TABLE XII

ELUTION OF NUCLEOSIDES, NUCLEOTIDES, AND NITROGENOUS BASES IN BIO-GEL P-2 CHROMATOGRAPHY

Compound	Elution Volume, ml[a]
ATP	275
ADP	310
5'-AMP	360
3'-AMP	374
3',5'-Cyclic CMP	369 (elutes with 3',5'-cyclic GMP when present)
2'-Deoxy-3',5'-cyclic AMP	383
3',5'-Cyclic IMP	389
3',5'-Cyclic AMP	402
2',3'-Cyclic AMP	418 (elutes with 3'-AMP when present)
3',5'-Cyclic GMP	431-444
Adenosine	456
Adenine	493

[a]These values varied ±3 ml between different elutions except for 3',5'-cyclic GMP as shown.

APPENDIX IX

TERMINATION OF CYCLIC AMP DEGRADATION BY HEAT AND TRICHLOROACETIC ACID

The reactions for the degradation of cyclic AMP were for 10 minutes with termination either by adding 1.0 ml of 6% TCA or by heating reaction tubes in a boiling water bath for 3 minutes. The data are summarized in Table XIII. The callus source was the 12,100 × g pellet from a loblolly pine callus homogenate.

TABLE XIII

TERMINATION OF CYCLIC AMP DEGRADATION

Incubation	Net Tritium cpm from TLC	
	Cyclic AMP	Adenosine
Reaction I (TCA termination)	2400	179
Control I (TCA terminated at t = 0)	2872	16
Reaction II (heat terminated)	2016	262
Control II (heat terminated at t = 0)	2580	16

APPENDIX X

TLC SEPARATION OF [8-^{3}H]-CYCLIC AMP DEGRADATION PRODUCTS

[8-^{3}H]-Cyclic AMP degradation products were chromatographed on thin-layer plates of cellulose and of silica gel. The results are shown in Fig. 31 and 32, respectively.

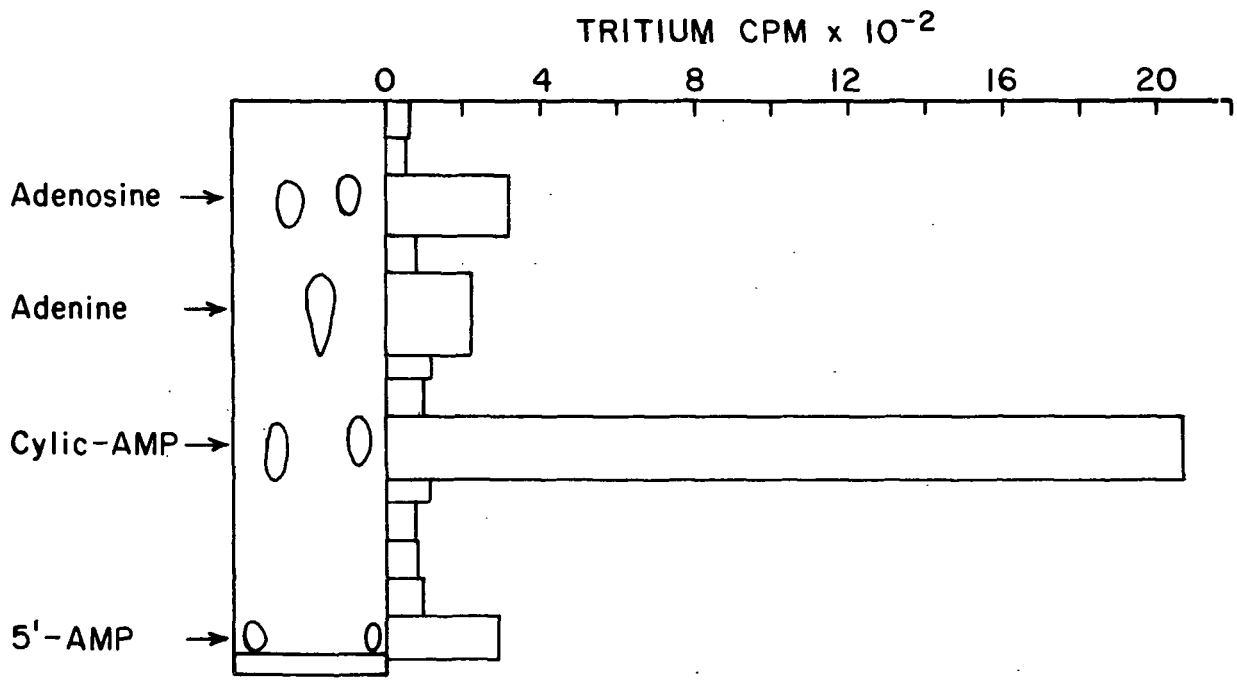


Figure 31. TLC Separation of Cyclic AMP Degradation Products on Cellulose.

MN Polygram $Cel_{300}UV_{254}$ Coated Plates with n-Butanol-Methanol-Ethyl Acetate-Concentrated Ammonium Hydroxide (7:3:4:4, v/v) Developer (49)

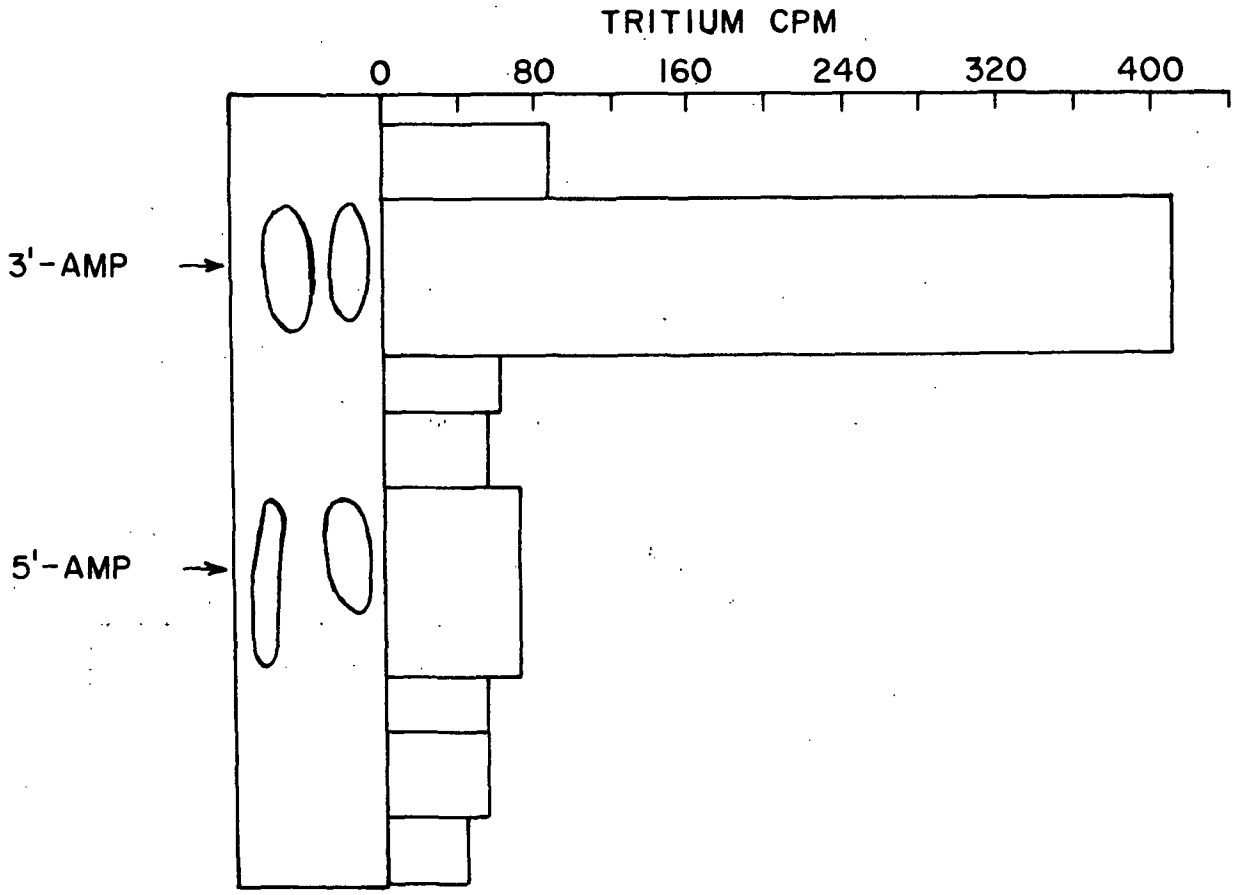


Figure 32. TLC Separation of Cyclic AMP Degradation Products on Silica Gel.

Silica Gel F-254 Coated Plates with Propanol-2-Concentrated Ammonium Hydroxide-Water (70:15:15, v/v) Developer

APPENDIX XI

SYNTHESIS OF ATP FROM ADENINE BY PINE CALLUS

The results shown in Fig. 33 represent a 2-cm strip cut from the right edge of a 20 × 20 cm TLC sheet following development in two directions (D_1 and D_2) with n-butanol-methanol-ethyl acetate-concentrated ammonium hydroxide (7:3:4:4, v/v). Because of their greater mobility in the first developing solvent, 3',5'-cyclic AMP, 2',3'-cyclic AMP, adenosine, and adenine standards were moved to the left of the 2-cm wide strip. The strip was then developed a third time (D_3) using propanol-2 -concentrated ammonium hydroxide-water (70:15:15, v/v). The UV absorption is from an ATP standard solution.

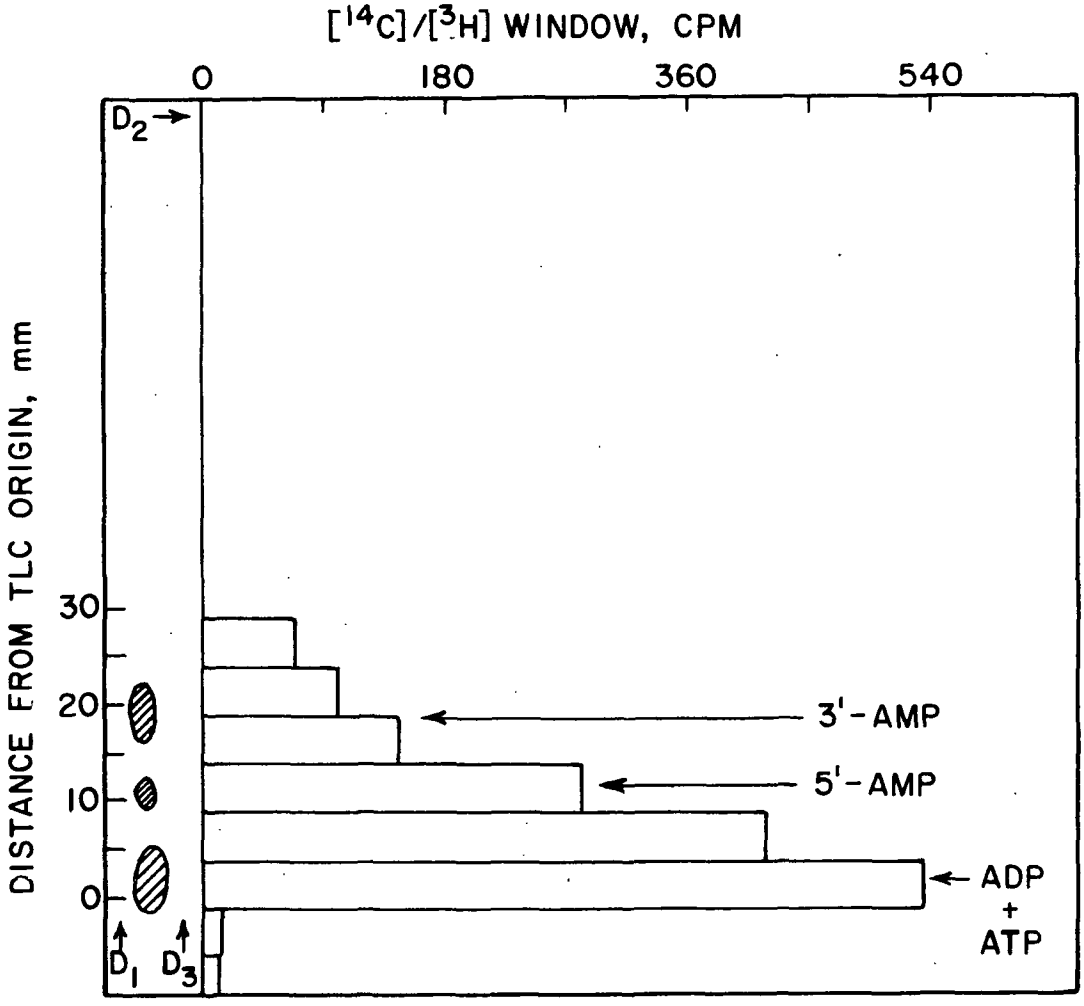


Figure 33. [^{14}C]-ATP Synthesized from [8-^{14}C]-Adenine by Loblolly Pine Callus

APPENDIX XII

SIMULTANEOUS TRITIUM AND CARBON-14 COUNTING OF BIO-GEL P-2 ELUATE BY LIQUID SCINTILLATION

	Channel 1[a], cpm	Channel 2[b], cpm
Cocktail D only	45.0	10.0
[8-^{3}H]-Cyclic AMP standard in 1M citrate (pH 4.4)	10739	10.0
[8-^{14}C]-5'-AMP standard in 1M citrate (pH 4.4)	8176	13476

[a] Channel 1 = [^{3}H] only isoset.

[b] Channel 2 = ^{14}C/^{3}H window.

% [^{14}C] in [^{3}H] window:

$$\frac{8131 \text{ cpm}}{8131 + 13466 \text{ cpm}} = 37.6\%$$

Calculation of [^{14}C] and [^{3}H]:

Channel 1 = 182.2 cpm

Channel 2 = 227.2 cpm

Total [^{14}C] cpm = (227.2-10.0)/0.624 = 348 cpm.

[^{14}C] cpm in Channel 1 = 348-217 = 131 cpm.

Total [^{3}H] cpm = 182-45-131 = 6 cpm.

The [^{3}H] counting efficiency was 26-30%.

The [^{14}C] counting efficiency was 67-69%.