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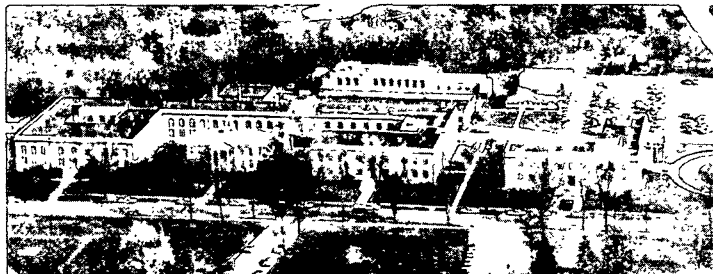

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cAMP IN LOBLOLLY PINE CALLUS

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INTRODUCTION

Propagation of trees by tissue culture would benefit the forest industry. Although formless masses of plant tissue — called callus — can be cultured, their development into recognizable plant structures is unpredictable. The Institute of Paper Chemistry is currently engaged in substantial research aimed at understanding the events that underlie the growth of conifer callus into organs like roots and shoots and eventually into whole plants. An important part of that effort consists of biochemical probing at the molecular level. The thesis research reported in this paper constitutes a valuable adjunct to the broader biochemical investigations.

Many metabolic processes in multicellular organisms (including trees) are regulated by organic compounds called hormones. The common plant hormones are generally different in molecular structure from the common animal hormones, but they are typical hormones, synthesized in one part of a living system in very small amounts and transported elsewhere in the system to exert their effects. At least some hormones have been found to be indirect agents, i.e., their sites of action are not the metabolic reactions themselves but rather are points at which they influence the concentration of a mediator which in turn may or may not be a direct agent. Biochemical transformations ordinarily proceed in many small steps rather than in one tremendous leap.

The compound cyclic adenosine monophosphate (cAMP) is well established as a mediator of hormonal control in animals; its role in plants and, in fact, its very existence in plants is now a very controversial issue. We sought evidence for the presence of cAMP and enzymes involved in its metabolism in laboratory-cultured loblolly pine cells.

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Evidence obtained in this study suggests that cAMP does exist in loblolly pine callus tissue and that its concentration fluctuates with increasing callus growth rate. It was demonstrated that a substance extracted from the callus competes strongly with authentic radioactive cAMP for a binding site on a cAMP-binding protein.

This paper is being submitted for publication in Plant Physiology.

Evidence for the Presence of Cyclic Adenosine 3':5'-Monophosphate and Its Metabolism in Loblolly Pine (*Pinus taeda* L.) Callus[1,2]

Richard H. Smeltzer[3] and Morris A. Johnson

ABSTRACT

Evidence is presented suggesting the occurrence of cAMP and adenyl cyclase activity in loblolly pine (*Pinus taeda* L.) callus cultures grown aseptically on a defined medium. The presence of enzymes in the callus capable of catalyzing cAMP hydrolysis also was detected. A direct relationship was found between apparent cAMP concentration and the rate of fresh-weight increase of dark-grown callus during a six-week passage. Autoradiography and 8-^{3}H-cAMP recovery indicated that exogenously supplied cAMP may be selectively bound in callus cell nuclei. The synthetic auxin, 2,4-D, was found to limit 8-^{3}H-cAMP uptake by callus cells, but it did not influence the distribution of radioactivity between the nucleus and the cytoplasm.

INTRODUCTION

The role of cAMP as second messenger in the action of some animal hormones and its role in regulating carbohydrate utilization by bacteria have been well documented and often cited. These findings have stimulated numerous investigations of the presence of cAMP in a wide variety of higher plants as well as studies of its ability to mimic plant hormones in their effects on physiology and metabolism. The presence of cAMP has been reported in bean (4), maize (2), and oat (28) seedlings, oat coleoptiles (22, 26), lettuce seeds and cabbage leaves (24), and in cultured tissue from soybean (3), tobacco, carrot, and sycamore (24). The cAMP concentrations reported in these foregoing studies varied from 11 to 890 pmoles/g fresh weight.

Niles and Mount (20) failed to detect cAMP in broad bean tissue although, using the Gilman assay, a positive response was registered which they concluded to be the result of binding competition by 2':3'-cAMP[4]. Amrhein (1) recently pointed out possible pitfalls in the use of assays for cAMP based on binding competition with ^{3}H cAMP for regulatory sites of a cAMP-dependent protein kinase. He used the protein kinase activation assay for cAMP to reinvestigate a number of the reports of cAMP in higher plants mentioned above and reported upper limits of possible cAMP concentrations from 0.3 to 25 pmoles/g fresh weight. The results of this work were interpreted as casting doubt on the presence of cAMP in higher plants. Ownby et al. (21) have investigated [^{14}C] metabolites from 8-^{14}C-adenosine which cochromatographed with cAMP and concluded that the concentration of cAMP in oat coleoptiles was probably no greater than 7 to 11 pmoles/g fresh weight. These recent reports suggest that for the plant materials reexamined, the concentration of cAMP first reported were probably high.

Neither the presence of cAMP nor of enzymes capable of regulating its concentration has been investigated in cultured cells of coniferous tree species. This report presents evidence for the presence of cAMP, adenyl cyclase activity, and cAMP hydrolytic activity in loblolly pine callus.

MATERIALS AND METHODS

Callus Cultures. *Pinus taeda* L. callus used in this study was initiated from 1971 branch cuttings from trees grown in North Carolina. The callus was initiated June 14, 1971, on agar medium NBA-10 (30). Callus which formed was subcultured back to medium NBA-10 and grown on this medium in the dark at 27 C with subculturing at three-week intervals prior to use in this study.

Callus Extraction, Extract Preparation, and cAMP Assay. Callus to be assayed for cAMP was removed from the agar and quickly immersed in liquid nitrogen, then powdered with a stainless steel pestle and beaker precooled to -196 C. The powdered callus was extracted with 5 ml of ice-cold 6% trichloroacetic acid per g of callus powder in the presence of 8-^{3}H cAMP (3 to 4 x 10^{3} cpm, specific activity 21.8 Ci/mmole, purchased from International Chemical and Nuclear Corp.). Following sedimentation of solids at 21,600 *g* (15 min at 2 C), the trichloroacetic acid was removed from the supernatant by extracting five times with water-saturated ethyl ether, two supernatant volumes each time. Residual ether was then removed from the water layer by heating the extracts for three min in a water bath.

Following lyophilization, the extracts were redissolved in 1.0 ml of 8.3 mM tris-HCl, pH 7.5, and chromatographed using neutral alumina columns as described by White and Zenser (29) except that the equilibration and elution buffer was changed to 8.3 mM tris-HCl, pH 7.5. This modification did not change the cAMP elution volume as reported by White and Zenser. Eluate fractions containing

putative cAMP were combined, lyophilized, and redissolved with 4 mM Na_2EDTA in a volume which resulted in a sixfold concentration of alumina eluate. Fifty-µl aliquots were counted by liquid scintillation to estimate 8-^{3}H-cAMP recovery. Samples to be treated with 3',5'-cNPDE before assay were redissolved in distilled water and Na_2EDTA was added after 3',5'-cNPDE treatment.

Fifty-µl aliquots of callus extracts prepared as described above were assayed using a cAMP binding protein kit purchased from Amersham/Searle Corp. The cAMP binding protein supplied was prepared from bovine muscle as described by Gilman (11). The assay was carried out as suggested by Amersham/Searle Corp. except that 100 µl aliquots of the assay supernatant were counted for tritium activity rather than the recommended 200 µl following the removal of unbound ^{3}H-cAMP with activated charcoal.

Adenyl Cyclase Assay. Adenyl cyclase activity was assayed in callus cell suspensions and in both fractions resulting from sedimenting a callus homogenate at 600 g for 10 min. The homogenizing and assay buffers were modified from those used by Wood et al. (31). Cell suspensions were prepared by placing callus in two volumes (w/v) of 50 mM tris-HCl, pH 7.5, containing 5 mM $MgCl_2$ and dispersing the cells by agitation in a Dubnoff shaking incubator at 30 C for one hr prior to use in the assay. Callus homogenates were prepared in the same buffer using a TenBroeck homogenizer. Homogenates were centrifuged at 600 g, the pellet was resuspended in an equal volume of homogenizing buffer, and 100 µl aliquots of either callus cell suspension or homogenate fraction were used in each reaction. The total reaction volume was brought to 150 µl by adding 50 mM tris-HCl, pH 7.5, containing 15 mM $MgCl_2$, 90 mM NaF, 3 mM dithiothreitol, and 6 mM Na_2ATP. Control reactions were run (a) eliminating Na_2ATP and (b) eliminating NaF. cAMP accumulation during these reactions was monitored using the cAMP binding

protein assay. Standard curves were prepared with buffered cAMP solution identical, except for natural extract components, to the reaction mixture being assayed.

Cell-free Preparations. All crude enzyme preparations in this work were made at 2 to 4 C. Protein was measured using the microbiuret method reported by Koch and Putnam (16). The turbidity correction was made by their method of subtracting twice the absorbance at 392 nm from the absorbance at 330 nm. All enzyme reactions were run in the Dubnoff incubator at 30 C and quenched by heating reaction tubes in a water bath.

3',5'-cNPDE Reactions. The 3',5'-cNPDE preparation used to examine the cAMP binding protein assay response was purchased from Sigma Chemical Co. and was prepared from beef heart by the procedure of Butcher and Sutherland (5) through the DEAE-cellulose step. Preparations from Sigma lots 120C-7740 and 120C-7751-9 were used in this study. Conditions for specific reactions are reported in the Results.

Endogenous Enzymatic cAMP Degradation. The activities of enzymes catalyzing cAMP hydrolysis were assayed using a chromatographic method similar to the one reported by Wood et al. (31). Callus was prepared for assay either as a cell suspension or centrifuge fractions from callus homogenates. The reaction and homogenizing buffers were the same as those used by Wood et al. (31). Fifty-µl of callus preparation (34 to 56 µg protein) was used in each assay and 8-^{3}H-cAMP (30,000 cpm) was supplied in assay buffer to bring the total reaction volume to 120 µl. Following reaction termination, the solids were removed by centrifugation and reaction products in the supernatant were separated by TLC. In reactions containing callus whole cells, the reaction mixtures were sonicated for 15 min with a General Electric Ultrasonic Generator at 150-175 mamp to rupture cells after reaction termination and before sedimenting the solids.

Fifty-µl aliquots of reaction supernatants were chromatographed using 5 x 20 cm MN Polygram Cel_{300} UV_{254} coated TLC plates (Brinkmann Instrument Co.) which were developed with n-butanol-methanol-ethyl acetate-28% NH_4OH (7:3:4:4, v/v). Following development of the TLC plates and detection of standard adenine derivatives (by UV absorption), sections of the thin-layer coating were scraped into vials for tritum counting.

Liquid Scintillation Counting. All counting was done with a Beckman LS-100 liquid scintillation counter. Samples were in 10 ml Beckman cocktail D (100 g naphthalene and 5 g PPO per liter solution in 1,4-dioxane). When counting tritiated compounds in TLC scrapings, 0.5 ml methanol was added to the vial containing the coating before cocktail D was added. The TLC scrapings containing ^{3}H-cAMP were counted within one hr of cocktail addition since tritiated activity was significantly quenched in these samples at longer times. The tritium counting efficiency from TLC scrapings was 26 to 30%.

Callus Growth Measurement. For growth measurements, 10 pieces of callus in one set were weighed and returned to the agar medium each week. Two additional sets of 10 pieces each were selected for both fresh and oven-dry (42 C for 21 hr) weights. One set was weighed at the beginning of the six-week growth passage and the dry weight:fresh weight was 0.036. The other set was weighed at the end of the six-week passage and the dry weight:fresh weight was 0.050, which was identical to the ratio at six weeks for callus weighed and returned to the agar weekly during the growth passage. Four samples of two callus pieces each were selected for cAMP assay weekly. Callus weighed for growth rates and that selected for cAMP assay came from the same clonal subculture.

RESULTS

Examination of the cAMP Binding Protein Assay Response. Table I and Figures 1 and 2 show data obtained in the investigation of the cAMP binding protein assay response from loblolly pine callus extracts. Table I shows that authentic cAMP can be assayed accurately in the presence of the loblolly pine callus extract as well as in the presence of the additives for the 3',5'-cNPDE reaction. When the callus extract was diluted 1:1 with assay buffer, the apparent cAMP concentration indicated by the assay was reduced approximately as expected. Using 3',5'-cNPDE reaction conditions reported in Table I (excessive for the apparent cAMP amounts present), assay responses resulting from authentic cAMP and the callus extract were depressed in a similar manner. Figure 1 shows the results of assaying a callus extract at several dilutions where the undiluted extract was the equivalent of 1.23 g callus fresh weight. The apparent cAMP concentration from the assay was not proportional to the extract dilution until quantities less than the equivalent of 0.3 g callus fresh weight were assayed.

The results suggest that some component of the callus extract, which coelutes with cAMP from alumina, competes equally with cAMP for the assay protein binding sites (at proper extract dilution). After treatment with 3',5'-cNPDE, this extract component no longer competes with cAMP for binding sites on the assay protein. Compounds known to be susceptible to degradation catalyzed by the Sigma 3',5'-cNPDE preparation (7) and also known to elute from alumina with or near cAMP (29) are 2',3'-cAMP and cGMP.

To distinguish among these three possibilities, 3',5'-cNPDE reactions were run for short times (pH 7.5, 30 C) with a low enzyme concentration (0.0126 unit/250 µl) and the reactions were monitored via the binding protein assay as shown in Figure 2. The V_{max} reported by Sigma for the 2',3'-cAMP phosphodiesterase

activity associated with their 3',5'-cNPDE preparation was 0.006 that reported for the 3',5'-cNPDE activity with cAMP as the substrate. With the reaction conditions reported in Figure 2, 2',3'-cAMP degradation should have been negligible, assuming that the K_m of the two enzymes for their cyclic nucleotide substrates are the same order of magnitude. Figure 2 shows that the decrease in assay response with time during 3',5'-cNPDE treatment approximately paralleled that of a cAMP solution of similar concentration. The solid circles indicate amounts of cAMP expected to remain at various reaction times as calculated from the integrated Michaelis equation (6). The insert to Figure 2 shows that 8-^{3}H-cGMP (40 nM) was hydrolyzed at greater than twice the rate of 40 nM 8-^{3}H-cGMP in the first 30 sec of reaction with the same conditions as reported in Figure 2.

These results suggest that, of the compounds considered as possibilities for the component of loblolly pine callus extracts which was responsible for the assay response, cAMP is the most likely.

cAMP Fluctuation During a Six-Week Growth Passage. cAMP concentrations and fresh weight increases of the callus were monitored during a six-week growth cycle in the dark and the results are illustrated in Figure 3. On the basis of a callus dry weight:fresh weight of 0.05, apparent cAMP concentrations in the callus varied over the six-week period from 35 to 125 pmoles/g fresh weight. The cAMP concentration maxima at 2 and 5 weeks after subculture were concomitant with accelerations in the rate of callus fresh weight increase.

Adenyl Cyclase Activity. An accumulation of cAMP with time was indicated in incubations containing the 600 g supernatant from a callus homogenate, but very little, if any, accumulation was observed with the 600 g pellet fraction. The rate of cAMP accumulation during the first 30 min of reaction was 0.033 pmole/min/mg protein, in the presence of 30 mM NaF (Fig. 4). When NaF was omitted, no net

accumulation of cAMP was observed during 60 min incubations. cAMP hydrolysis catalyzed by enzymes from the callus was strongly (90%) inhibited by 10 mM NaF. Enzymatic cAMP hydrolysis apparently dominated in loblolly pine callus homogenates resulting in no net cAMP accumulation from adenyl cyclase reactions unless degradation was inhibited. The small increase in cAMP during the first 30 min in the control reaction with ATP omitted from the reaction medium may result from the availability of endogenous ATP (unpublished results) as substrate for the adenyl cyclase of the callus. No ATP regenerating system was used and, as might be expected, cAMP levels were reduced between 30 and 60 min. When whole callus cells were assayed for adenyl cyclase activity, a slow, steady accumulation of cAMP was indicated for at least 90 min.

cAMP Hydrolysis. As shown in Figure 5, adenosine was the most stable product of 8-^{3}H-cAMP degradation by the callus. This result suggested the presence of nucleotidase activity in the callus, which was confirmed by using 8-^{14}C-5'-AMP as the substrate (unpublished results). Adenine increased at a very slow rate during cAMP degradation and a small amount of AMP was present, although constant with incubation time. An isotope trapping experiment indicated that 3'-AMP and 5'-AMP were present in a ratio of 3:1. These observations with crude preparations or whole callus cells appeared to be similar to results obtained by others investigating cAMP degradation in plants of agricultural interest (3, 9, 17, 18, 27, 31). The lag period of at least 15 min in 8-^{3}H-cAMP degradation by the callus _in vivo_ suggests that cAMP is taken into the callus cell before its degradation begins. This observation is consistent with those of Giannattasio _et al_. (10) and Gordon _et al_. (12), but unlike the observations of Brewin and Northcote (3) on cAMP degradation in soybeans.

The results of investigating various sedimentation fractions from a loblolly pine callus homogenate for cAMP hydrolytic activity (Table II) indicated that this activity was concentrated in the 12,000 *g* pellet. These data are from a preparation in which the solids sedimenting at 600 *g* (10 min) were not removed. The removal of these heavier solids from the 12,000 *g* pellet preparation resulted in an increase in the specific degradative activity of this fraction from 0.9 mmoles to 1.4 mmoles 8-^{3}H-adenosine produced per mg protein.

Autoradiography. Autoradiographic localization of ^{3}H-activity centers following incubation of loblolly pine callus with 8-^{3}H-cAMP for 2 hr indicated that 70% of the radioactivity was located in cell nuclei (Table III). Auxin supplied as 2.3 μm 2,4-D did not influence the distribution of radioactivity between the nucellei and the cytoplasm. The rate of 8-^{3}H-cAMP uptake by the callus in the presence of 2,4-D was only about one-half the rate in the absence of 2,4-D as estimated by the total number of ^{3}H-activity centers observed.

DISCUSSION

The primary purpose of this work was to investigate the possible presence of cAMP and enzymes capable of controlling its concentration in aseptically grown callus cultures of loblolly pine. Having found evidence for the presence of cAMP in these cultures, it was of interest to determine how the apparent cAMP concentration fluctuated during a six-week growth cycle, in the dark, following subculture.

Evidence supporting the authenticity of the cAMP binding assay as applied to loblolly pine callus extracts included: (a) an accurate assay of authentic cAMP in the presence of the callus extract, (b) reduction of the assay response proportional to extract dilution (indicating binding competition from the extract

equivalent to that of cAMP) and (c) on treatment with a 3',5'-cNPDE preparation, a decline in the assay response at a rate consistent with cAMP hydrolysis but inconsistent with the degradation rates of other compounds expected to be present following chromatography of the extract through alumina. The 3',5'-cNPDE preparation used was similar to the preparation used by Butcher and Sutherland (5) to characterize cAMP in human urine. Substances were present in callus extract preparations which significantly reduced the rate of 3',5'-cNPDE catalyzed cAMP hydrolysis if extracts from the equivalent of more than 0.15 g callus fresh weight were treated.

The dilution of the partially purified callus extract was found to be critical for accurate cAMP estimation by the binding assay. If the equivalent of more than 0.3 g callus fresh weight per 50 µl was assayed, the cAMP indicated was low (see Fig. 1). This is thought to be the result of some component in the extract which enhanced cAMP binding to the protein kinase preparation. The observable result of the binding enhancement was an increase in the amount of 8-^{3}H-cAMP bound to the kinase.

The cAMP concentration indicated in the dark-grown callus cultures fluctuated in a direct relationship with changes in the rate of callus fresh weight increase. A possible parallel to these results is the report of Kamisaka _et al_. (13) that exogenously supplied N^{6}, 2'-O-dibutyryl-cAMP and 2,4-D acted synergistically in stimulating the fresh weight increase of Jerusalem artichoke tuber slices. The cause and effect relationship between cAMP concentrations and the rate of fresh weight increase in loblolly pine callus was not investigated; however these results suggest that cAMP may play a role in water uptake by the callus.

The apparent cAMP concentrations varied over a broad range depending on the physiological condition of the callus and the time from subculture. Callus pieces which were obviously darker in color than most at 1 to 3 weeks after subculture appeared to contain higher cAMP concentrations at 3 weeks than the lighter callus. Considerably more variation in cAMP concentrations was observed between samples of the darker callus than was observed in the lighter callus. Overall results suggest that cAMP levels in loblolly pine callus fluctuate rather than remain constant and relatively high cAMP concentrations are expected during periods of acceleration in the rate of callus fresh weight increase.

Adenyl cyclase activity was detected in the callus by measuring cAMP accumulation with the binding assay. The marked increase in the rate of cAMP accumulation when ATP was supplied exogenously suggests that the cAMP which accumulated was synthesized from ATP. In view of the finding that cAMP degradative activity was concentrated in the 12,000 g pellet, cAMP accumulating in the presence of the 600 g supernatant was a net amount in excess of that hydrolyzed. In contrast to assays of broken cell preparations, a very small linear accumulation of cAMP for at least 90 min was indicated in the assay of callus whole cells. The presence of adenyl cyclase and cAMP degradative activities in the unorganized loblolly pine callus renders possible the participation of cAMP in developmental processes in loblolly pine.

cAMP degradative activity observed in loblolly pine callus was similar in most respects to that observed by others investigating cAMP degradation in plants. Few investigators have attempted to localize this activity within the cell; however, Brewin and Northcote (3) have reported up to 35% of the c-NPDE activity in soybean callus to be associated with the plasmalemma. With loblolly pine callus, it appeared that very little cAMP hydrolytic activity was associated

with the plasmalemma; rather, up to 70% of the activity was associated with the 12,000 g pellet with little or no activity in the 100,000 g pellet.

The observations on the effect of auxin treatments and the location of radioactivity in the callus following its incubation with 8-^{3}H cAMP were not conclusive, but suggest areas of further study which could lead to an understanding of cAMP action in loblolly pine callus. The recovery of 8-^{3}H-cAMP doubled in incubations where the solids sedimenting at 600 g were removed from the 12,000 g solids. The observation that label was concentrated in callus nuclei following incubation with 8-^{3}H-cAMP was a further indication that cAMP binds to nuclear components. Other workes have reported that cAMP plays a role in DNA synthesis (14, 15) in RNA synthesis (25), and in the synthesis of specific enzymes (8, 19, 23). The observed effect of 2,4-D in limiting cAMP uptake by the callus was similar to the report of Gordon *et al*. (12) on the effect of auxin on cAMP uptake by maize coleoptiles.

Since the gymnosperms are more primative than the angiosperms, their regulators of growth and development may not be identical. This is the first published report of the investigation of cAMP and its metabolism in gymnosperms. The results of this work suggest that further investigations into the role of cAMP in regulating loblolly pine callus metabolism are warranted and further research is anticipated at this institution.

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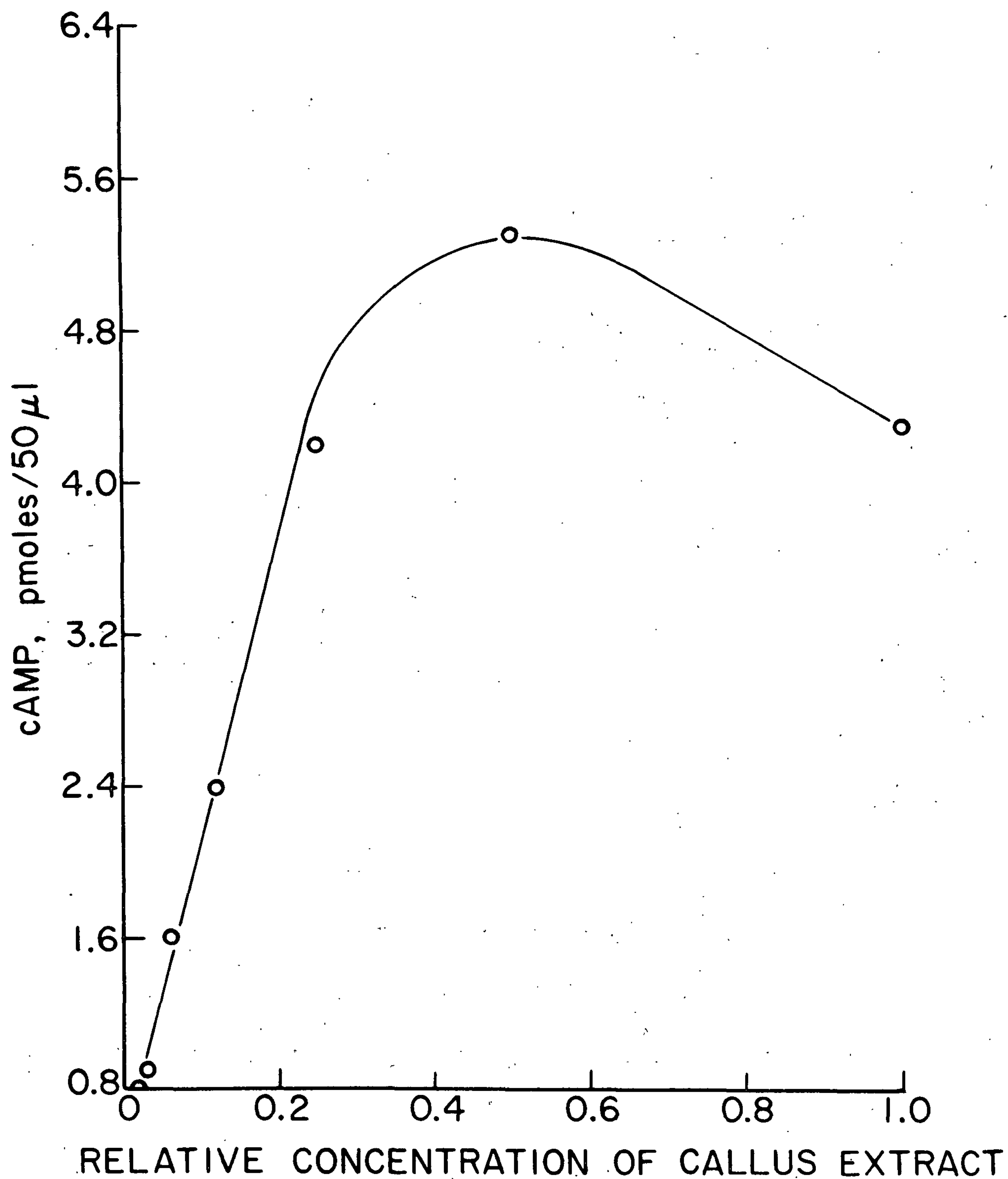

Fig. 1. cAMP concentration in loblolly pine callus extract at several dilutions determined by cAMP binding protein assay. Experimental points are single determinations.

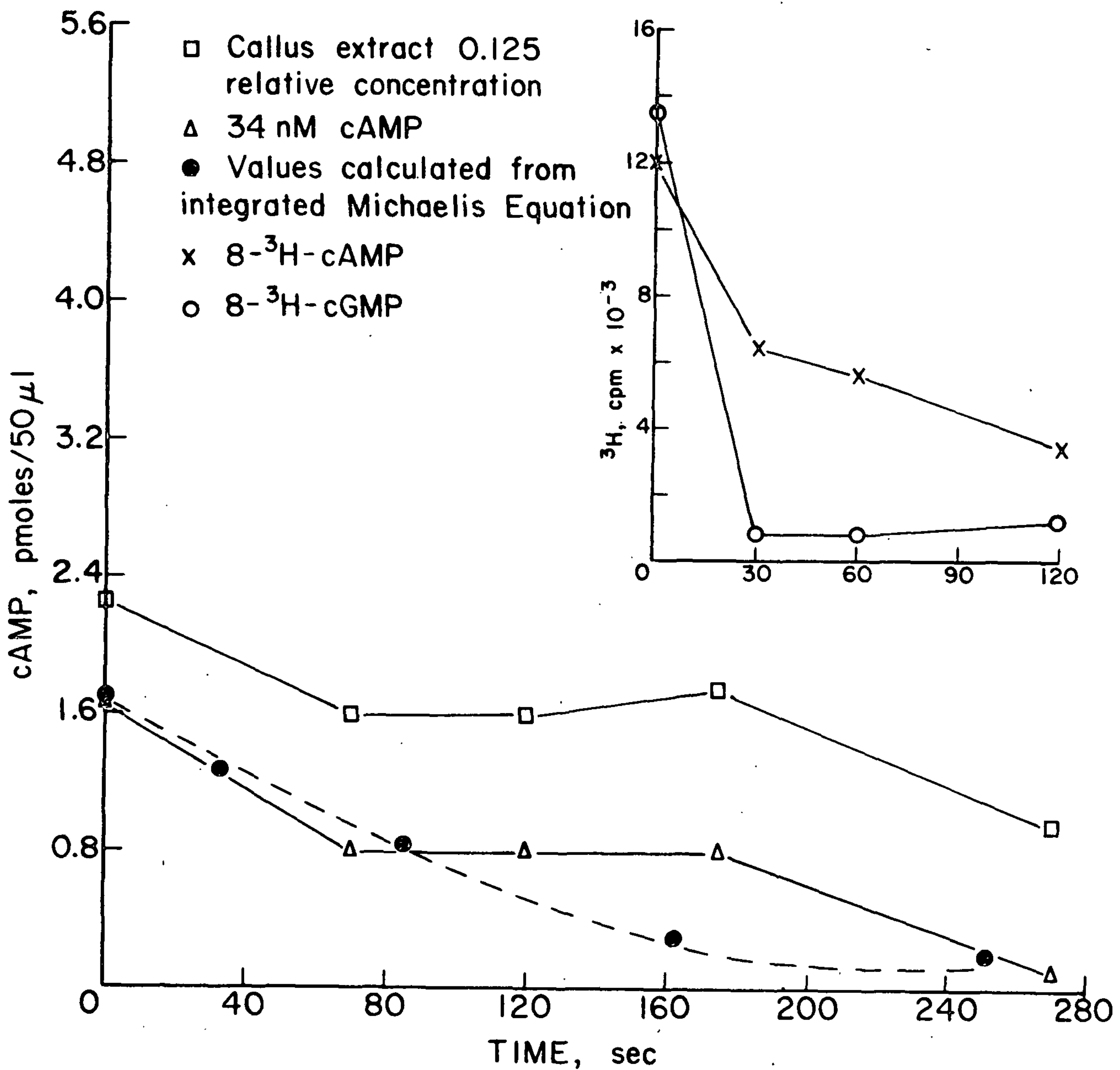

Fig. 2. Change in cAMP concentration with 3',5'-cNPDE treatment time. Reactions were monitored by cAMP binding protein assay. For reaction conditions, see text. Experimental points are averages of duplicate determinations.

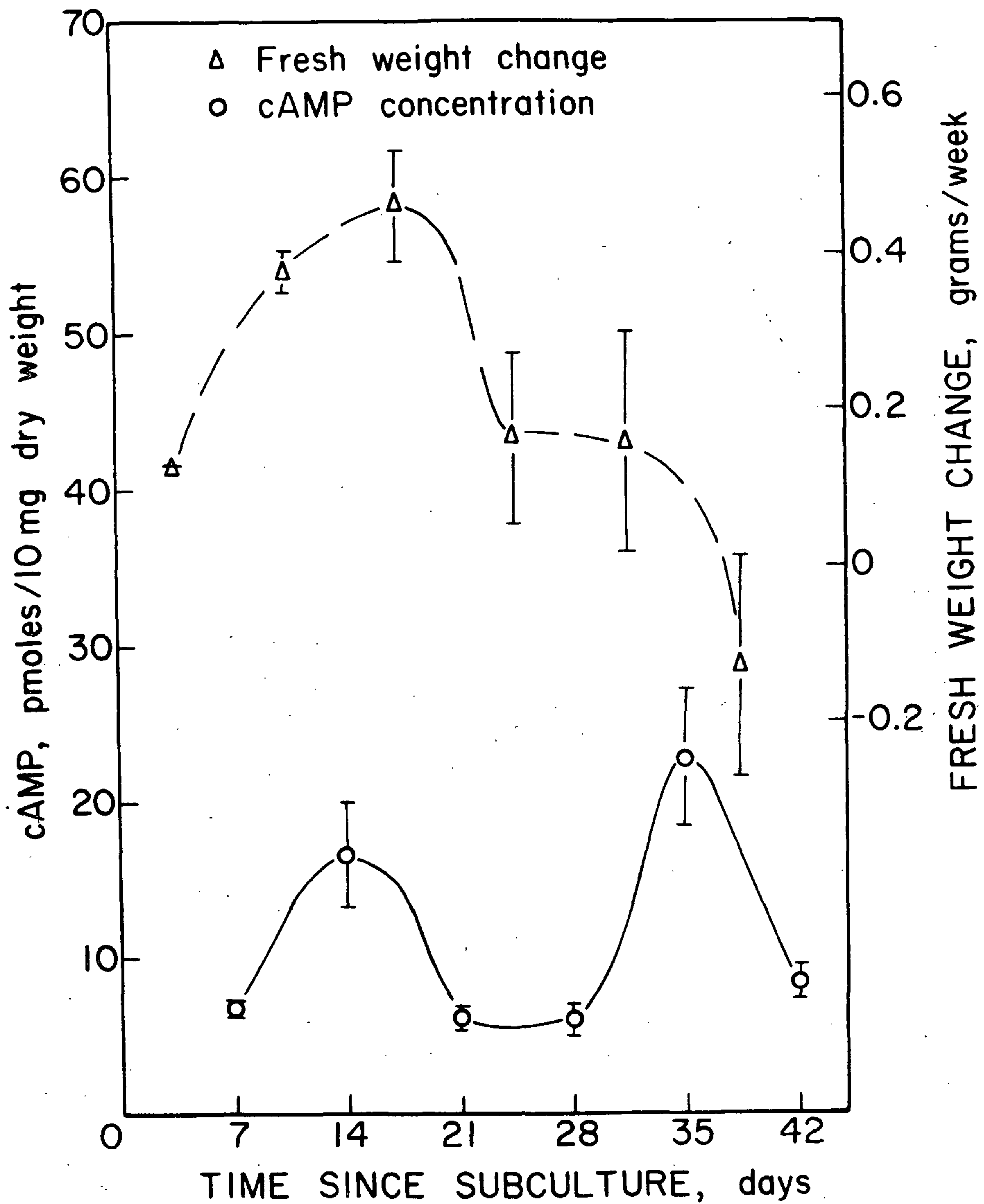

Fig. 3. Fluctuation in cAMP concentration and fresh weight growth of loblolly pine callus with time. Bars represent ± SE of ten observations of fresh weight growth and eight cAMP assays.

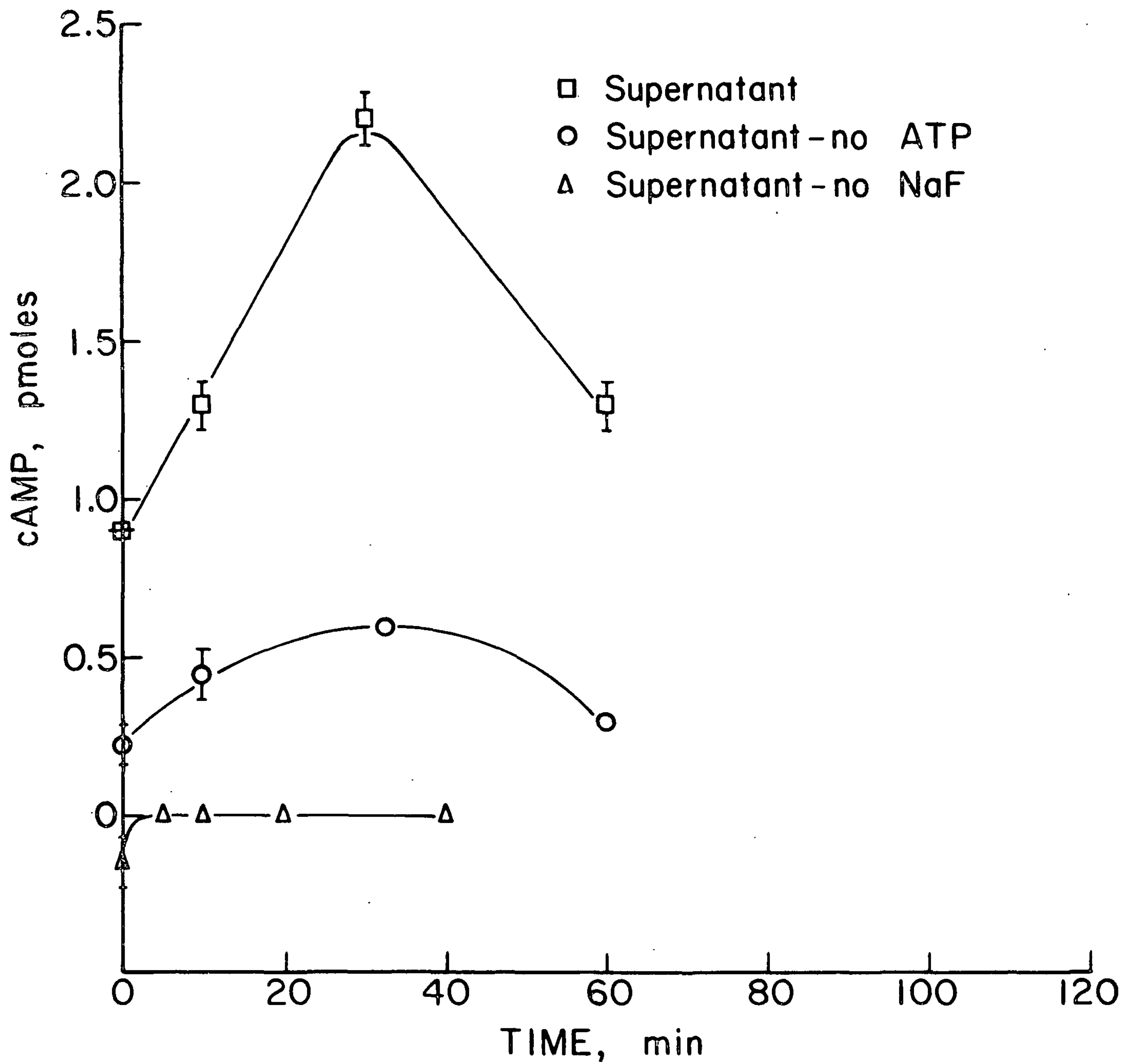

Fig. 4. cAMP accumulation with time in adenyl cyclase assay of a 600 g supernatant from a loblolly pine callus homogenate. Bars represent ± SE of three observations, four observations in controls.

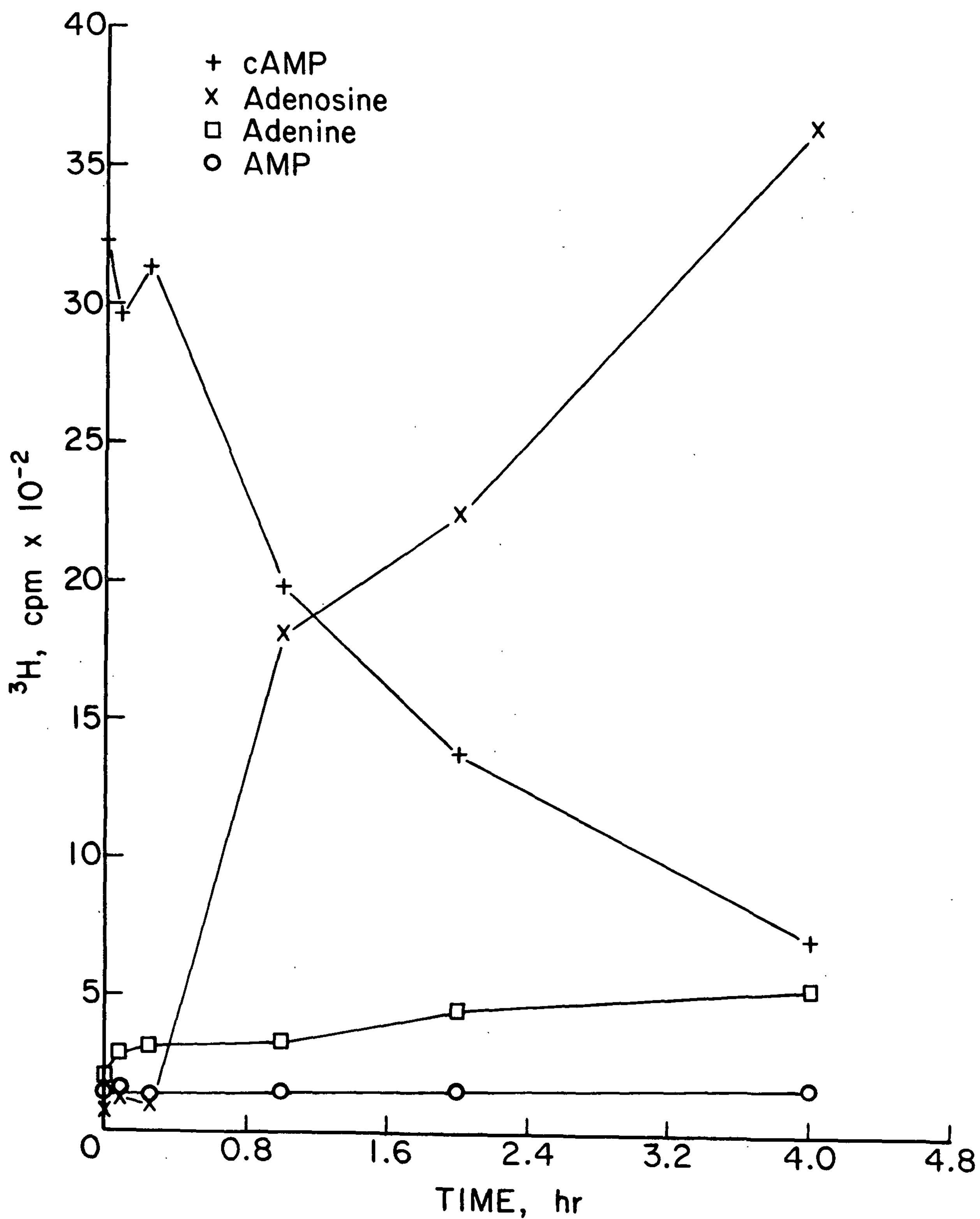

Fig. 5. 8-^{3}H-cAMP degradation *in vivo* by loblolly pine callus. Experimental points are averages of duplicate determinations.

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[2]This report is taken from a dissertation submitted by R. H. Smeltzer in partial fulfillment of the requirements for a Ph.D. degree at The Institute of Paper Chemistry, Appleton, Wisconsin.

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[4]Abbreviations: 2',3'-cAMP: cyclic adenosine 2':3'-monophosphate; 3',5'-cNPDE: 3':5'-cyclic nucleotide phosphodiesterase; cGMP: cyclic guanosine 3':5'-monophosphate; V_{max}: velocity maximum.