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Studies on the Presence of Adenosine Cyclic 3′:5′-Monophosphate in Oat Coleoptiles[1]

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ABSTRACT

The incorporation of adenosine-8-^{14}C into adenosine cyclic 3′:5′-monophosphate in coleoptile-first leaf segments of *Avena sativa* L. was investigated. Homogenates of segments incubated in adenosine-8-^{14}C for either 4 or 10 hours were partially purified by thin layer chromatography followed by paper electrophoresis. A radioactive fraction, less than 0.06% of the ^{14}C present in the original homogenate, migrated as adenosine cyclic 3′:5′-monophosphate during electrophoresis. Upon treatment with cyclic nucleotide phosphodiesterase, however, less than 10% of this radioactive fraction appeared as 5′-AMP. Deamination with $NaNO_2$ as well as further chromatographical purification also suggested that only a small fraction of the ^{14}C in the partially purified samples could be in adenosine cyclic 3′:5′-monophosphate. The data suggest that levels of this nucleotide can probably be no greater than 7 to 11 picomoles per gram of fresh weight in oat coleoptiles. Treatment of such coleoptiles with physiologically active concentrations of indoleacetic acid, furthermore, had no significant effect on the ^{14}C radioactivity in marker adenosine cyclic 3′:5′-monophosphate-containing fractions at any stage of purification during several experiments.

In a single experiment, no labeled guanosine cyclic 3′:5′-monophosphate could be detected in oat coleoptile-first leaf segments incubated in guanosine-8-^{14}C either with or without indoleacetic acid. These results do not support the hypothesis that a cyclic nucleotide mediates the action of indoleacetic acid on oat coleoptile extension.

In the last 5 years numerous attempts have been made to determine whether or not cAMP[3] exists in higher plants. Levels of cAMP ranging from 0.06 to 0.50 μM (approximately 60–500 pmoles/g fresh wt) have been reported from lettuce seeds, *Acer* cells, and tobacco and carrot pith tissue using both a protein kinase activation assay (20) and a bioluminescence assay (19, 20) and from *Robinia* phloem sap using a radioisotope dilution test (4). Brown and Newton (6) claim to have detected cAMP spectrophotometrically in purified preparations from *Phaseolus vulgaris* L. seeds, while cAMP was reported to occur in oat coleoptiles and soybean suspension cultures from assays utilizing a cAMP-binding protein (5, 27). The protein binding assay was also used by Kessler and Levinstein (16), who reported levels of cAMP ranging from 50 to 9500 pmoles/g fresh wt in various storage, stem, and leaf tissues. Using another approach, a radioactive compound having some of the properties of cAMP was reportedly isolated from maize coleoptiles (12), oat coleoptiles (24, 27), Bengal gram seeds (3), mustard seedlings (13), and barley aleurone layers (18) incubated in ^{3}H- or ^{14}C-adenine. Keates (15), on the other hand, was unable to demonstrate conclusively the occurrence of such a compound in barley aleurone layers incubated in ^{14}C-adenosine. Alvarez *et al.* (1) were also unable to confirm the presence of cAMP in barley seeds.

The status of cAMP in higher plants thus remains uncertain. Many assays developed for routine determinations of cAMP levels in animals may lack the high specificity required for measurement of cAMP in plant tissues, since plants possess compounds capable of interfering with such assays (unpublished data of Ray Bressan in our laboratory). Furthermore, because cAMP must occur in very low concentrations, very rigorous purification is required to establish its synthesis from radioactive precursors.

We report here our efforts to isolate labeled cAMP and cGMP from auxin-treated oat coleoptile-first leaf segments incubated in radioactive adenosine or guanosine. The difficulties in obtaining a pure compound and establishing that the substance is the cyclic nucleotide in question are the major points of emphasis.

MATERIALS AND METHODS

Preparation of Coleoptiles. Oat seeds (*Avena sativa* L. cv Park or Russell) were planted in trays of moist vermiculite and grown for 4 days in darkness at about 26 C. Two adjacent 5-mm subapical segments were cut from each coleoptile, the uppermost cut being made 3 mm below the tip. The segments were pooled in sterile distilled H_2O and subsequently distributed into incubation flasks.

Incubation Procedures. In experiment 1, 100 coleoptile segments were incubated on a water shaker bath in the dark for 10 hr at 29 C in a medium containing 5 mM potassium phosphate (pH 6.1), 0.5 mM $CaCl_2$, 50 μg/ml chloramphenicol, 0.5 mM theophylline (Sigma Chemical Co.), 0.01 mM IAA, and

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[3] Abbreviations: cAMP: adenosine cyclic 3′:5′-monophosphate; cGMP: guanosine cyclic 3′:5′-monophosphate; cIMP: inosine cyclic 3′:5′-monophosphate; PDE: cyclic nucleotide phosphodiesterase.

30 μCi of adenosine-8-^{14}C (Schwarz-Mann, 52.4 mCi/mmole). Final volumes were 4 ml.

In experiment 2, the incubation flasks contained 2.5 mM potassium phosphate (pH 6.0), 2% (w/v) sucrose, 50 μg/ml of chloramphenicol, 15 μCi of adenosine-8-^{14}C (Schwarz-Mann, 52.4 mCi/mmole) and either 0.01 mM IAA or sterile H_2O in a final volume of 4 ml. Each of 6 flasks received 75 coleoptiles and was incubated in the dark at 29 C for 4 hr.

In experiment 3, the incubation medium consisted of 5 mM potassium phosphate (pH 6.1), 50 μg/ml chloramphenicol, 1 mM $CaCl_2$, 0.01 mM theophylline, 15 μCi of guanosine-8-^{14}C (International Chemical and Nuclear Corp., 54.9 mCi/mmole) and either 0.01 mM IAA or sterile H_2O in a final volume of 3 ml. Incubation procedures were as in experiment 2.

In all experiments, glassware and all chemicals except the radioisotopes were sterilized by heat or by filtration. Subsequent dilution plating of incubation media on a National Institutes of Health agar medium (28) just prior to tissue analysis indicated approximately 10^3 bacteria/ml in experiments 1 and 2, while none were detected in experiment 3.

Chromatography Solvent Systems. Solvent systems used to separate cyclic nucleotides from contaminants were: isopropanol-NH_4OH-H_2O(7:1:2, v/v), solvent A; $(NH_4)_2CO_3$-acetone-H_2O(0.98 g:75 ml:30 ml), solvent B; isobutyric acid-NH_4OH-H_2O(57:4:39, v/v), solvent C; ethanol-1 M ammonium acetate (7:3, v/v), solvent D; methanol-1 M ammonium acetate (7:3, v/v), solvent E.

Extraction and Purification of Labeled Nucleotides. Coleoptile segments were rinsed in running tap water for 30 sec to wash off unabsorbed nucleosides, then either homogenized immediately (experiments 1 and 2) or frozen in liquid nitrogen (experiment 3). A Polytron homogenizer (Brinkmann Instruments, Inc.) was used for grinding the tissues. The homogenizing medium (at a temperature about 2 C) was 3 ml of 0.2 N $HClO_4$ containing 1 μmole of unlabeled cAMP or cGMP (experiment 3) as an internal marker. Authentic ^{3}H-cAMP (8×10^4 cpm in experiment 1 and 8×10^5 cpm in experiment 2) or ^{3}H-cGMP (4×10^4 cpm in experiment 3) were added just before homogenizing the tissues to monitor recoveries and purities of cyclic nucleotide fractions. Both ^{3}H-labeled cyclic nucleotides were obtained from the International Chemical and Nuclear Corp.

Homogenates were centrifuged at 27,000g for 20 min at about 5 C: supernatant fractions were then neutralized with 2.5 N KOH. After removal of $KClO_4$ by centrifugation, either all (experiment 1) or half (experiments 2 and 3) of each $HClO_4$-soluble fraction was chromatographed on a 20 × 20 cm MN 300 cellulose plate (Brinkmann Instruments, Inc.) in solvent A or solvent B. Cyclic nucleotide-containing regions detected under UV radiation were eluted with H_2O. Aliquots were taken from the resulting solutions for ^{3}H or ^{14}C analysis, and the remainder of each solution was dried with forced air. Radioactive products were redissolved in H_2O and subjected to paper electrophoresis on Whatman 3MM papers in 50 mM ammonium acetate (pH 6.5) for 8 to 10 hr in a cold room at 1 C with a voltage gradient of 40 v/cm. The zones of the resulting electropherogram were assayed for ^{3}H and ^{14}C by liquid scintillation spectrometry. The cyclic nucleotide-containing zones were then eluted with toluene, dried, and eluted with H_2O. The resulting aqueous solutions were air-dried, redissolved, and further purified by techniques varying with the experiment. Specific procedures are described below and in the "Results" section.

Hydrolysis with Phosphodiesterase. Samples (100 μl) partially purified by the above TLC and electrophoretic procedures were incubated for 1 to 3 hr with 2 μmoles of $MgSO_4$ and 0.1 unit of beef heart cyclic nucleotide PDE obtained from Sigma Chemical Co. Final volumes were 0.25 ml. Reactions were terminated by boiling, and denatured protein was removed by centrifugation.

Deamination. Samples subjected to the same two initial purification steps were partially deaminated by modifying the method of Kaplan (14) so that the reaction mixtures contained 136 mg of $NaNO_2$ and 0.35 ml of 2 N acetic acid in a final volume of 0.5 ml. The samples were incubated 48 hr at room temperature. In attempts to use the much higher $NaNO_2$ concentration and shorter reaction time employed by Kaplan, excess salt always prevented any effective separation by TLC or electrophoresis.

Measurement of the Specific Radioactivity of ATP in Coleoptile Segments. To determine the extent of isotopic dilution of adenosine-8-^{14}C during its conversion to ATP, oat coleoptile-first leaf segments were incubated under conditions similar to those described for experiments 1 and 2. Each of four flasks containing 50 segments (about 0.35 g fresh wt) received 14.5 μCi of adenosine-8-^{14}C (Schwarz-Mann, 47.3 mCi/mmole) in 2 ml of incubation medium. After 4 hr and 10 hr of incubation, tissues were harvested and homogenized in $HClO_4$ as described above. Following centrifugation and neutralization, the acid-soluble extract was concentrated by freeze-drying and duplicate samples of each were chromatographed two-dimensionally on Whatman 3MM paper using solvents C and E. Each ATP-containing region was located by autoradiography using Kodak Blue Brand x-ray film and an exposure period of 1 week, then eluted with H_2O. Four aliquots of each eluate were analyzed for ATP by the luciferase assay essentially as described by Ebadi *et al.* (8). For the determination of radioactivity, similar aliquots were spotted on paper and counted by liquid scintillation spectrometry. That the ^{14}C present was indeed in ATP was verified by chromatography with a 1.2 M LiCl solvent and sheets of PEI cellulose (Brinkmann Instruments).

Measurement of Radioactivity. Radioactivity from ^{3}H and ^{14}C was measured directly in zones from papers or TLC plates. These zones were placed in 7 ml of scintillation fluid containing 4 g/liter PPO and 50 mg/liter POPOP. Radioactivity was measured with a Nuclear-Chicago Unilux liquid scintillation spectrometer. Engberg plots (17) were used to determine correct settings for double label counting. Background radiation was measured by analyzing blank zones of equivalent sizes from paper or cellulose powder.

RESULTS

Purification of Cyclic Nucleotides by TLC and Electrophoresis. Thin layer chromatography in either solvent A or B eliminated most of the noncyclic nucleotides (R_F 0.0 to 0.10) but did not completely separate cAMP or cGMP (R_F 0.40 to 0.45) from nucleosides and bases (R_F 0.2 to 0.6). These were eliminated by the electrophoresis step, as indicated by results from experiment 1 shown in Figure 1. Contaminants in zones 2 to 11, which account for 90 to 95% of the total ^{14}C on the papers, consisted largely of adenine and adenosine as indicated by electrophoretic data not shown. The ^{14}C peak which roughly corresponded to the marker ^{3}H-cAMP peak centered at zone 17 contained approximately 1200 cpm of 6.9×10^6 cpm in the original $HClO_4$-soluble extract. The first two purification steps thus eliminated over 99.9% of the labeled contaminants. Similar purifications were obtained in experiments 2 and 3.

Analysis of Products Resulting from PDE Treatment in Experiment 1. Aqueous solutions were obtained by eluting the marker cAMP-containing regions of electrophoretic papers

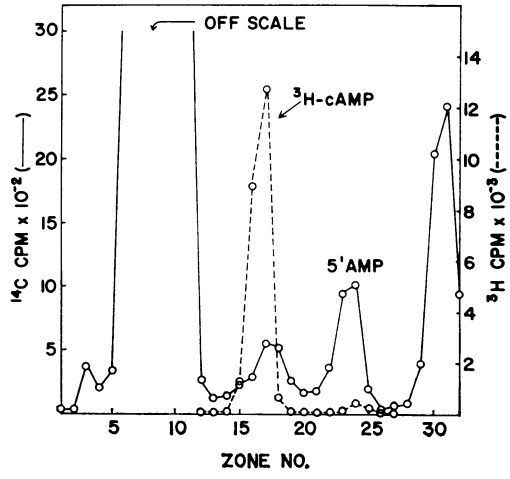

FIG. 1. Typical radioactivity profiles on electrophoresis papers showing the separation of cAMP from purine bases and nucleosides (zones 2 to 11). Prior to electrophoresis samples had been partially purified by TLC in solvent A. The off-scale region contained 105,500 cpm.

with H_2O. Half of the sample (100 μl) was subsequently treated with PDE, and reaction mixtures were then chromatographed one-dimensionally in solvent A alongside the sample half not treated with PDE. Typical results are shown in Figure 2. The PDE treatment converted 94% of the ^{3}H-cAMP to ^{3}H-5'-AMP, but the ^{14}C activity in the 5'-AMP region (zones 1–3) increased by only 24 cpm (from 2.8% to 6.6% of the total ^{14}C in that lane of the TLC plate). The major portion of ^{14}C activity was not affected by PDE and migrated similarly to cAMP (zones 10–12).

After correcting for incomplete hydrolysis, loss of marker cAMP, and the fact that only half the sample was hydrolyzed, the amount of ^{14}C present in cAMP can be no more than 161 cpm compared to 8.1×10^6 cpm of ^{14}C in the original acid-soluble extract.

Careful inspection of the data from the unhydrolyzed half-sample shown in Figure 2 reveals that the major peak in ^{14}C activity appears slightly beyond the ^{3}H-cAMP peak on the TLC plate. That the major part of this ^{14}C activity cannot be in cAMP was further demonstrated by additional chromatographical purification of the unhydrolyzed control sample of Figure 2. When chromatographed in solvent D (Fig. 3), the major peak of ^{14}C activity again migrated slightly beyond authentic ^{3}H-cAMP. Another contaminant (zones 28–30) was completely resolved from cAMP by this purification.

In preliminary work similar to that described above, 40% of the labeled material migrating as cAMP during TLC and electrophoresis migrated as 5'-AMP in solvent A after treatment with the same PDE preparation used in experiment 1. Additional chromatography in solvent D, however, suggested that the precursor to this 5'-AMP might not be cAMP. The possibility exists that in the preliminary work a labeled compound was produced in the tissues which was converted to 5'-AMP by a contaminant of the PDE preparation no longer active during experiments 1 and 2. Goldberg *et al.* (10) reported that PDE preparations purified 40-fold from beef heart contain contaminants which liberate 5'-AMP from ADP-ribose. In addition, AMP was also released from NADH, NAD, and NADP by the enzyme preparations. These compounds, however, are largely eliminated by our first purification step, TLC in solvent A.

Analysis of Products Resulting from Deamination Treatment. A second sample from experiment 1 was partially purified by TLC and electrophoresis, and then half of the sample was partially deaminated as described in "Materials and Methods." Both the deaminated and control half-sample were then subjected to paper electrophoresis. Figure 4 shows the radioactivity obtained in each zone of the resulting electropherogram. In the deaminated sample half, a small peak in ^{14}C activity appeared (zones 11–13) which chromatographed with authentic ^{3}H-cIMP, the product of deamination of ^{3}H-

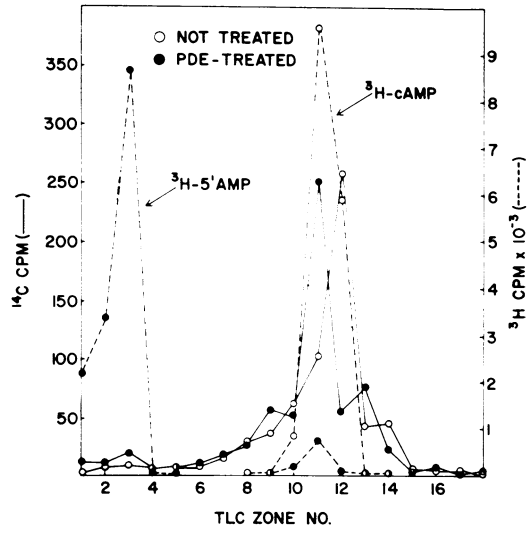

FIG. 2. ^{14}C (from metabolized ^{14}C-adenosine) and ^{3}H (from marker ^{3}H-cAMP) radioactivity profiles obtained by chromatography of PDE-treated and untreated half-samples in adjacent lanes of a TLC plate in solvent A. Samples used in the PDE treatments were obtained from the marker cAMP-containing region of electrophoresis papers in experiment 1. Since salts and residual protein in the PDE reaction mixture usually retarded chromatographic migration, it seems likely that the ^{14}C peaks shown in zones 11 and 13 of the PDE-treated half-sample represent the same compounds seen in zones 12 and 14 of the control half-sample.

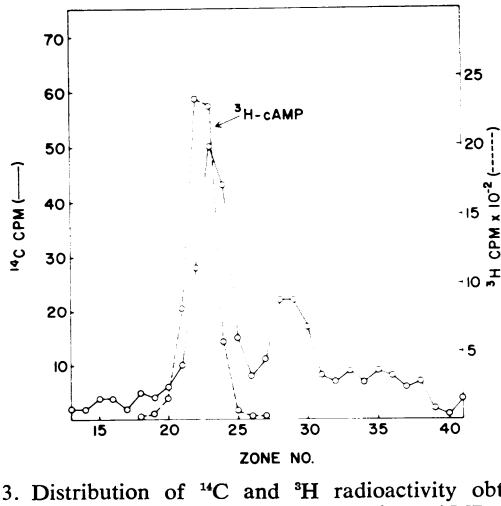

FIG. 3. Distribution of ^{14}C and ^{3}H radioactivity obtained by descending paper chromatography. The marker cAMP-containing region of the chromatogram (see Fig. 2, zones 10 to 12) containing the half-sample not treated with PDE was eluted with toluene to remove PPO and POPOP, then eluted with water. The resulting aqueous solution was concentrated with forced air and chromatographed on Whatman 3 MM paper in solvent C for 24 hr.

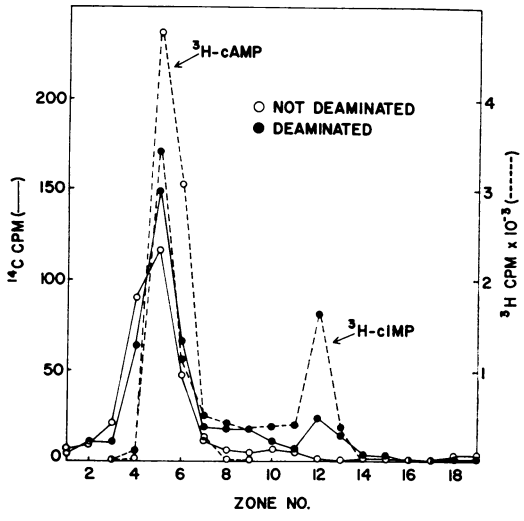

FIG. 4. Distribution of ^{3}H and ^{14}C radioactivity following paper electrophoresis of a control half-sample and a half-sample subjected to deamination (experiment 1). Both half-samples were spotted on adjacent lanes of Whatman 3 MM paper and subjected to electrophoresis for 5 hr with 50 mM ammonium formate (pH 3.0) as buffer.

cAMP. Deamination converted 30% of the marker ^{3}H-cAMP to ^{3}H-cIMP, but only about 11% of the ^{14}C on the electropherogram migrated with cIMP. These results therefore suggest that no more than 35 to 40% of the ^{14}C activity in the major peak of the undeaminated half-sample can be cAMP.

In two preliminary experiments, the major radioactive product of deamination migrated between cAMP and cIMP when chromatographed in solvent C. We thus cannot say with certainty that deamination of our samples resulted in the production of any cIMP from cAMP.

Analysis of Products of PDE Hydrolysis in Experiment 2. Samples subjected to TLC in solvent A followed by electrophoresis were also hydrolyzed with PDE, but were then chromatographed in solvent D. The amount of ^{14}C migrating with 5'-AMP again was almost undetectable. Figure 5 shows the distribution of ^{3}H (from authentic labeled cAMP) and of ^{14}C (from metabolized adenosine) in each zone. Also shown is the distribution of the two isotopes in corresponding zones resulting from simultaneous chromatography of an equivalent amount of unhydrolyzed sample in an adjacent lane of the same Whatman 3MM paper. PDE treatment resulted in nearly complete (96%) conversion of ^{3}H-cAMP into ^{3}H-5'-AMP. However, most of the ^{14}C in the same solution was now distributed among three contaminants, one still migrating with authentic cAMP and two beyond it. Whether the barely detectable peak of ^{14}C corresponding to 5'-AMP truly represents PDE-catalyzed conversion of ^{14}C-labeled cAMP could not be established. It seems likely that one of the contaminants (zones 30–32) is the same as that found in experiment 1 (see zones 28–30 of Fig. 3).

Table I summarizes the results of experiment 2. Following step 3, samples were either chromatographed in solvent D (3a) or treated with PDE and then chromatographed in solvent D (3b). Recovery of cAMP after each step was measured by means of authentic ^{3}H-cAMP added to the homogenizing medium. The ^{14}C activity in the cAMP and 5'-AMP fractions has been corrected for loss and for splitting the samples after steps 1 and 3. Thus, the calculation of 44 cpm in 5'-AMP after step 3b was based on a ^{14}C peak having less than 20 cpm (see zones 15–18, Fig. 5).

Measurement of the Specific Radioactivity of ATP in Oat Coleoptiles. Two-dimensional chromatography of the neutralized acid-soluble extract revealed that the major products of metabolized adenosine-8-^{14}C are ATP and two other compounds which have not yet been identified. The specific radioactivity of ATP reached 11.4 ± 1.4 mCi/μmole (25% that of precusor adenosine-^{14}C) after 4 hr and 21.3 ± 7.6 mCi/μmole

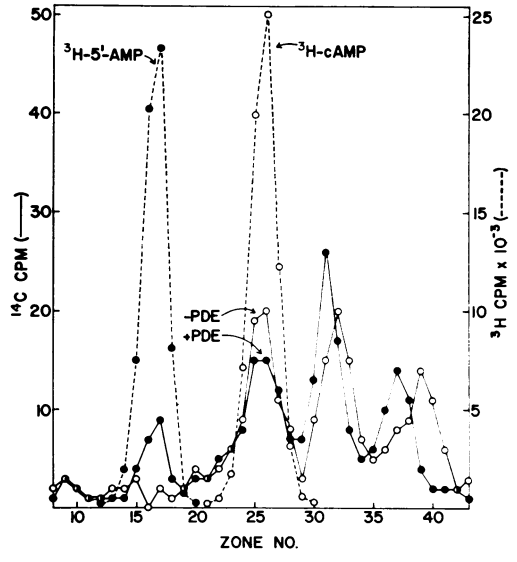

FIG. 5. ^{14}C and ^{3}H radioactivity profiles obtained by paper chromatography of a partially purified sample following treatment of half of the sample with PDE. Results shown are from a pooled sample obtained from auxin-treated tissues in experiment 2. Samples from control tissues not treated with PDE gave results similar to those of the unhydrolyzed half-sample. Spillover of ^{3}H into the ^{14}C channel was less than 0.007%.

Table I. *Summary of Purification of Oat Coleoptile Homogenates in Experiment 2*

All analyses of ^{14}C and ^{3}H were made at comparable counting efficiencies. After step 1, half of each sample was frozen, and the remainder was used in subsequent purification steps. Following step 3, the three half-samples from control tissues were pooled, as were the three half-samples from IAA-treated tissues. Half of each pooled sample was then used in steps 4a and 4b. The values given in the table have been corrected for sample pooling and splitting and for loss of cyclic AMP after each step as determined by the recovery of ^{3}H-cyclic AMP.

Purification Step	^{14}C Activity of Cyclic AMP Fraction		Recovery of Marker ^{3}H Cyclic AMP	
	Control	IAA	Control	IAA
	cpm		*%*	
1. $HC10_4$ extract[1]	1.65 × 10^6	1.85 × 10^6	100.0	100
2. TLC in solvent A	46,800	46,000	80.0	84.0
3. Paper electrophoresis	1,086	932	46.9	48.5
4. a. Paper chromatography in solvent D	362	318	31.5	30.2
b. PDE, then chromatography in solvent D		44[2]		29.8[2]

[1] Values represent all ^{14}C-labeled compounds.

[2] As 5'-AMP. The value of 44 cpm represents the net increase in ^{14}C in the 5'-AMP region after treatment with PDE.

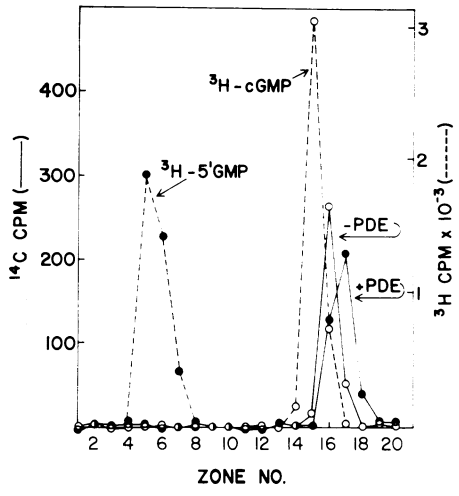

FIG. 6. Chromatographic distribution of ^{14}C (from metabolized ^{14}C-guanosine) and ^{3}H (from marker ^{3}H-cGMP) radioactivity in a partially purified cGMP sample following treatment of half of the sample with PDE. Chromatography: PEI-F cellulose thin layer plates developed in 0.1 M LiCl, dried, and redeveloped in 0.35 M LiCl.

(45% that of precursor adenosine) after 10 hr of incubation. The concentration of ATP in the tissues averaged 31.3 $\pm$ 8.7 $\times$ 10^{-6} mmoles/g fresh wt.

Purification of cGMP Fractions from Oat Coleoptiles. Homogenates of apparently sterile oat coleoptiles incubated in ^{14}C-8-guanosine were partially purified by TLC in solvent B and electrophoresis as described previously. A discrete peak usually coinciding exactly with marker UV-quenching and ^{3}H-cGMP was produced by electrophoresis. When a half-sample obtained from auxin-treated tissues was eluted from an electrophoresis paper and incubated with PDE, then developed alongside the untreated half on a TLC plate of PEI-F cellulose, no new ^{14}C peak corresponding to 5'-AMP was found (Fig. 6). The ^{14}C-labeled material which had migrated as cGMP during TLC and electrophoresis was not cGMP, since it migrated slightly beyond authentic cGMP on PEI cellulose and was not hydrolyzed by PDE under conditions which converted over 96% of the marker ^{3}H-cGMP in the same reaction tube to ^{3}H-5'-GMP. A control and an auxin-treated sample yielded essentially identical results.

The major portion of the unknown radioactive material which migrated with cGMP during TLC and electrophoresis appears to be xanthosine. Both xanthosine and the unknown material have very similar mobilities during chromatography in solvent C and when subjected to paper electrophoresis at pH 6.5. After an aliquot of the unknown material was boiled in 1.5 N HCl for 1 hr, a radioactive product was produced which, when chromatographed in solvent C, had the same mobility as xanthine, the product of acid hydrolysis of xanthosine.

Effect of IAA and cAMP on Oat Coleoptile Elongation. In experiments carried out under conditions similar to those described above, 0.01 mM IAA increased the length of 5-mm coleoptile segments by 35% in 4 hr, while control segments increased less than 3%. These results confirm that the auxin treatments used in all of our experiments did promote growth, a fact that was noted but not quantified with sections just prior to homogenization. Cyclic AMP (Sigma Chem. Co.) at 1 mM, either with or without 1 mM theophylline, did not promote elongation and had no significant effect on IAA-stimulated growth (unpublished results).

DISCUSSION

The data presented in experiments 1 and 2 indicate that, even after purification by TLC and electrophoresis, 65 to 90% of the ^{14}C-labeled material which migrates with ^{3}H-cAMP cannot be hydrolyzed to 5'-AMP by PDE or deaminated to cIMP by $NaNO_2$. These experiments illustrate the difficulty in obtaining pure cAMP fractions from plant tissues and suggest that contaminants very similar to cAMP are present. If these contaminants are chemically similar to cAMP, they might cause misleading results in various assays utilizing the binding of cAMP to proteins, especially when neither the binding protein nor the assay samples have been extensively purified.

For the purpose of discussion, the ^{14}C radioactivity in cAMP is assumed to be that which migrated with authentic ^{3}H-cAMP during chromatography and electrophoresis and appeared as a net increase in ^{14}C-5'-AMP after PDE hydrolysis. Thus, the ^{14}C in cAMP was 161 cpm in experiment 1 and 44 cpm in experiment 2 (values corrected for loss, incomplete hydrolysis, and splitting of the sample). Based on the results of a separate experiment, the specific radioactivity of ATP, the precursor to cAMP, was approximately 24.5 mCi/mmole in experiment 1 and 13.2 mCi/mmole in experiment 2. Assuming that the specific radioactivity of any tissue cAMP would be at or near that of ATP, the estimates of cAMP concentration are approximately 11 pmoles/g fresh wt from the results of experiment 1 and 7 pmoles/g fresh wt from experiment 2. These estimates are similar to those calculated for *Chlamydomonas* by Amrhein and Filner (2), and are 10 to 50 times lower than concentrations in higher animals (21, 26). Raymond *et al.* (20) calculated that cAMP concentrations of several plant tissues ranged from about 50 to 950 pmoles/g fresh wt. They obtained comparable results with the beef heart protein kinase-activation assay and with a bioluminescence (luciferase) assay. The latter assay involves hydrolysis of cAMP to 5'-AMP by PDE, then enzymatic conversion of 5'-AMP to ATP. Our preliminary results suggest that under certain conditions the nonspecificity of PDE preparations will cause greatly overestimated determinations of cAMP in the luciferase assay, at least for oat coleoptiles.

Since the low level of radioactivity (44–161 cpm) in cAMP precluded further attempts at purification, it is not certain that cAMP is synthesized at all in oat coleoptiles. These results are not consistent with an earlier report (24) of as much as 112,000 cpm in cAMP purified from IAA-treated oat coleoptiles incubated in adenine-8-^{14}C. Our results suggest that substances other than cAMP comprise a large portion of putative cAMP fractions reported in that and other previous studies (3, 18).

The proposal (24) that cAMP mediates auxin-induced elongation of oat coleoptiles presently appears highly questionable. In experiment 2 and several preliminary experiments with oat coleoptiles and soybean hypocotyl segments, we have never detected any increase in radioactivity of marker cAMP-containing fractions due to auxin treatment. Since the results of Keates (15) further indicate that cAMP is not synthesized in response to GA_3 in barley aleurone layers, we consider it unlikely that this nucleotide plays a ubiquitous second messenger role in higher plants. If cAMP is synthesized by them at all, it may function only in certain processes not universal to all species. The case may be analogous to that in bacteria, where cAMP is required for functioning of the lactose operon in *Escherichia coli* and other species (7), but it does not greatly affect *in vivo* expression of the galactose operon (22) and cannot be detected at all in some species (23, 25).

The single experiment with guanosine-8-^{14}C produced no evidence for cGMP synthesis in oat coleoptile segments. Assuming that little or no isotopic dilution occurred, the level of cGMP could probably be no more than 1 pmole/g fresh wt, a value 5 to 50 times lower than those reported for animals (9).

The numerous effects of exogenous cAMP (see ref. 11 for references) show that it can modify many plant physiological responses. Nevertheless, much additional work will be required to establish unequivocally the status of cyclic nucleotides in plants.

Note Added in Proof. Since this manuscript was submitted for publication, N. Amrhein (Planta 118:241–258, 1974) has shown, using a protein kinase activation assay, that cyclic AMP levels in oat coleoptile sections were less than 25 pmoles/g fresh wt in one experiment and less than 8 pmoles/g fresh wt in another. Three other species gave values less than 2 pmoles/g fresh wt.

Acknowledgment—The technical assistance of Patricia Fox is gratefully acknowledged.

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