Plant Physiol. (1976) 57, 29–37

Attempts to Detect Cyclic Adenosine 3′:5′-Monophosphate in Higher Plants by Three Assay Methods[1, 2]

Received for publication December 27, 1974 and in revised form April 17, 1975

RAY A. BRESSAN AND CLEON W. ROSS
Department of Botany and Plant Pathology, Colorado State University, Fort Collins, Colorado 80523

JOZEF VANDEPEUTE[3]
Department of Pharmacology, University of Colorado Medical Center, Denver, Colorado 80220

ABSTRACT

Endogenous levels of cyclic adenosine-3′:5′-monophosphate in coleoptile first leaf segments of oat (*Avena sativa* L.), potato (*Solanum tuberosum* L.) tubers, tobacco (*Nicotiana tabacum* L.) callus, and germinating seeds of lettuce (*Lactuca sativa* L.) were measured with a modified Gilman binding assay and a protein kinase activation assay. The incorporation of adenosine-8-^{14}C into compounds with properties similar to those of cyclic AMP was also measured in studies with germinating lettuce seeds. The binding assay proved reliable for mouse and rat liver analyses, but was nonspecific for plant tissues. It responded to various components from lettuce and potato tissues chromatographically similar to but not identical with cyclic AMP. The protein kinase activation assay was much more specific, but it also exhibited positive responses in the presence of compounds not chromatographically identical to cyclic AMP. The concentrations of cyclic AMP in the plant tissues tested were at the lower limits of detection and characterization obtainable with these assays. The estimates of maximal levels were much lower than reported in many previous studies.

The ubiquitous occurrence and diverse physiological roles of cAMP[4] in animal tissues have promoted considerable interest over possible analogous functions of this nucleotide in higher plant tissues. Apparently cAMP can elicit physiological responses in plants similar to those evoked by certain plant hormones. Cyclic AMP has been implicated in replacing GA_3 as in inductive agent (11). Claims that it can mimic the inductive effects of IAA (29, 30), cytokinins (30), and phytochrome (13, 26) exist, as well. The significance of such reports is often obscured by the use of high concentrations of cAMP, incomplete replacement of the hormonal effect, similar if not equal results obtained by using other adenine nucleotides, and failure to test 2′:3′-cAMP, a close analogue of cAMP not possessing the same biological activity in animal systems. The effects of exogenous cAMP on higher plants might be purely pharmacological. Conclusive evidence that it has a natural function in plants must await the unequivocal demonstration of endogenous cAMP.

Several reports claim the existence of endogenous cAMP in higher plants. Six techniques have been utilized in these studies: (*a*) isotopic labeling with a metabolic precursor of cAMP such as adenine or adenosine (24, 28), (*b*) assays using a cAMP-binding protein (2, 3, 15, 32), (*c*) the protein kinase activation assay (25, 27), (*d*) the radioimmunoassay (7, 9, 19), (*e*) the luciferase assay (25, 27), and (*f*) spectrophotometric measurement of the compound after isolation from *Phaseolus vulgaris* L. seeds (5). The levels of cAMP reported in these studies range between 50 and 9,600 pmoles/g fresh weight. Other estimates of cAMP levels are far less than the above values (1, 14, 21, 23). Frequently, authors using either the binding assay or radioimmunoassay have assumed that these assays possess a high level of specificity. This has led them to attempt to measure cAMP in crude homogenates with little or no purification. The reliance of these procedures on the displacement of labeled cAMP or cAMP derivative from a protein kinase subunit (binding protein) or antibody makes them susceptible to interference by salts or other impurities in the samples which could enhance displacement (4).

We will show that the binding assay can give erroneously high values for cAMP levels in various plant tissues even after rigorous purification of the extract. In lettuce seed extracts, some of these erroneous results can be attributed to a specific fraction chromatographically similar but not identical to cAMP. By utilizing isotopic labeling and the kinase activation assay, we found that the maximum level of cAMP in a variety of higher plants is much lower than previously reported by several authors.

MATERIALS AND METHODS

Measurement of Radioactivity. Radioactivity was measured with a Nuclear-Chicago Unilux-III liquid scintillation spectrometer. All measurements were made in 7 ml of a toluene scintillation fluid containing 4 g/l PPO and 50 mg/l POPOP except for Millipore filters used in the binding and kinase activation assays, which were analyzed in 5 ml of a 3:2 mixture of the toluene cocktail and methyl cellosolve. Radioactivity in zones of TLC plates or paper chromatograms was measured directly. Aliquots of solutions containing labeled compounds were spotted on paper or Millipore filters (HAWP) which were dried and then placed in scintillation fluid to measure radioactivity. Background values were determined by measuring appropriate blanks. Whenever

[1] This research was supported by National Science Foundation Grant GB 38625.

[2] This paper is dedicated to the memory of Donald W. Denna.

[3] Present address: Plant Science Research, Agricultural Division, CIBA-GEIGY Corp., Greensboro, N. C. 72409.

[4] Abbreviations: cAMP: cyclic adenosine 3′:5′-monophosphate; 2′:3′-cAMP: cyclic adenosine 2′:3′-monophosphate; PDE: beef heart cyclic nucleotide 3′:5′-phosphodiesterase. PEI: polyethyleneimine. EAA: ethanol-ammonium acetate; MAA: methanol-ammonium acetate; IAW: isopropanol-ammonia-water; IBA: isobutyric acid-ammonia-water.

dual label spectrometry was used, Engberg plots (18) were made to determine the correct instrument settings.

Chromatography Solvent Systems. Solvent systems used to separate labeled adenine nucleotides by MN 300 cellulose (Brinkmann Instruments, Inc.) TLC were: ethanol-1 M ammonium acetate (7:3, v/v), EAA: methanol-1 M ammonium acetate (7:3, v/v), MAA; isopropanol-NH_4OH-H_2O (7:1:2, v/v), IAW; isobutyric acid-NH_4OH-H_2O (57:4:39, v/v), IBA. Separations on PEI cellulose plates (Brinkmann Instruments, Inc.) were made using 1 M LiCl, while methanol-ethyl acetate-NH_4OH-1-butanol (3:4:4:7, v/v) was used on silica gel (Brinkmann Instruments, Inc.) plates. Paper electrophoresis was performed with Whatman No. 3 MM paper in 50 mM ammonium acetate (pH 6.4). Chromatographic materials used for column separations included Dowex 50 X8-200 (Sigma Chemical Co.), neutral alumina (Waters Associates, Inc.), PVP (General Aniline and Film Corp.), and DEAE-cellulose (Sigma Chemical Co.).

Chemicals. The following were purchased from Sigma Chemical Co.: ATP, ADP, AMP, 2′:3′-cAMP, cAMP, PDE, theophylline, chloramphenicol, and luciferase-luciferin (firefly tail extract). Both adenosine-8-^{3}H-cyclic 3′:5′-monophosphate (27 Ci/mmole) and [γ-^{32}P]ATP (16.6 Ci/mmole) were purchased from Amersham Searle Corp. Adenosine-8-^{14}C (51 mCi/mmole) was obtained from Schwarz/Mann. Calf thymus histone (f1) was a gift of Dr. Thomas Langan, University of Colorado Medical School.

Incubation and Homogenization of Lettuce Seeds. Twenty g of unimbibed (40% H_2O) lettuce (*Lactuca sativa* L. cv. Grand Rapids) seeds were aerated in the light with forced air in 700 ml of a chloramphenicol solution (50 μg/ml) for 8 hr with one change of solution at 3.5 hr. After 8 hr, the seeds were transferred to 100 ml of a solution containing 50 μg/ml chloramphenicol, 0.5 mM theophylline, and 100 μCi of adenosine-8-^{14}C and again aerated. Aliquots were removed periodically to determine uptake of ^{14}C-adenosine. After incubation in the ^{14}C-adenosine for 5.5 hr, the seeds were filtered through a metal screen and rinsed with ice-cold H_2O for 3 min. They were then homogenized in 100 ml of ice-cold 0.2 N $HClO_4$ containing 3 μCi (110 pmoles) of ^{3}H-cAMP. Homogenization was performed at maximum speed with a Polytron (Brinkmann Instruments Inc., PT-30 generator) for 2 min on ice with one 20 ml rinse of the generator and grinding vessel. The homogenate was filtered through cheesecloth, and the filtrate was centrifuged. (This and all other centrifugations were performed at 14,000*g* for 20 or 30 min at 4 C.) The supernatant was collected, neutralized with 2.5 N KOH, chilled on ice for 30 min, and centrifuged to remove $KClO_4$. This supernatant was freeze-dried, and the residue was redissolved in 12 ml of distilled H_2O and frozen.

Purification of ^{14}C-labeled Adenosine Lettuce Seed Extract. Half of the neutralized extract was purified by a series of four steps: two column chromatographic steps (neutral alumina followed by Dowex 50), a paper electrophoresis step, and a separation by descending paper chromatography in EAA. Both columns (2.7 × 15 cm) were eluted with H_2O to remove ^{3}H-cAMP marker and comigrating ^{14}C. Fractions from these columns were analyzed for ^{14}C and ^{3}H by spotting aliquots of each onto paper (Whatman No. 3 MM) and by measuring radioactivity by dual label spectrometry. The distribution of the isotopes on the electropherogram and paper chromatogram was determined by measuring ^{3}H and ^{14}C present in 1 cm strips. Column fractions containing ^{3}H-cAMP marker were collected, freeze-dried, and redissolved in a small volume of H_2O before further purification. Strips from the electropherogram containing ^{3}H-cAMP marker were rinsed six times in toluene to remove PPO and POPOP, eluted with three 20-ml volumes of 20% ethanol, air-dried, and redissolved in 0.2 ml of H_2O before EAA chromatography.

Specific Radioactivity of ATP. After elution of the alumina column with H_2O to remove ^{3}H-cAMP and comigrating ^{14}C, the same column was eluted with 225 ml of 0.2 M Na_2HPO_4 to remove ATP and determine its specific radioactivity. The Na_2HPO_4 fraction was freeze-dried, redissolved in 8 ml of water, chilled and centrifuged to remove excess salt, and fractionated on a Dowex 50 column with H_2O. After measuring the radioactivity in aliquots of each fraction, a single ^{14}C peak was detected. The pH of the material from this peak was adjusted to 7.5 with 0.1 N NaOH, and the volume was brought to 100 ml with distilled H_2O. A 0.1-ml aliquot was mixed with 0.06 μmole each of cAMP, AMP, ADP, and ATP and chromatographed by cellulose TLC in MAA. Distinct ^{14}C peaks corresponding to the ATP, ADP, and cAMP standards were present. (The presence of a ^{14}C peak resolved from any ^{3}H-cAMP in the previous alumina step but matching cAMP on this chromatogram seemed significant.) The radioactivity migrating with the ATP standard was used to calculate the specific radioactivity of ATP.

To determine the total ATP in an equivalent sample, 0.1-ml aliquots of the same Dowex 50 eluate were examined with the luciferase assay. The procedure used was similar to that of Ebadi *et al.* (8). The luciferase was prepared by redissolving the firefly extract (250 mg) of Sigma in 10 ml of ice cold water, chilling on ice for 3 hr, then centrifuging. The supernatant was frozen in 2-ml portions and an 8-fold dilution of this was used in the assay. Freezing and thawing significantly diminished the activity of the enzyme. It was necessary to stir the enzyme preparation continuously while adding it to the assay vials because of the formation of small particles possessing enzyme activity. The reaction mixture, containing 0.4 ml of 72 mM tris-Cl, pH 7.5, 0.1 ml of 3 mM $MgSO_4$, 0.1 ml of luciferase, and 0.1 ml of either sample or ATP standard, was added directly to scintillation vials. The reaction was started by the addition of the luciferase. Luminescence was determined quantitatively by counting in the scintillation counter with the coincidence circuit turned off. Ten successive 6-sec counts were made 15 sec after the addition of the enzyme. The mean of the last three counts was used as a measure of each sample's activity. A plot of this average against pmoles of ATP was linear from 0 to 200 pmoles of ATP. Samples from the purified lettuce seed extract were measured with and without 50 pmoles of ATP to examine the possibility of luciferase inhibitors in the extract. No inhibition was detected. These data were used along with the measurement of radioactivity in the ATP fraction to estimate the specific radioactivity of ATP.

Characterization of a ^{14}C-labeled Compound(s) Similar to cAMP. The ^{14}C-labeled fraction co-chromatographing with cAMP in MAA during ATP purification described above was partially characterized by additional chromatography, by the effect of PDE on it, and by its ability to react in the binding assay. A 20-ml portion of the 100 ml of eluate from the Dowex 50 column known to contain the unidentified ^{14}C-compound(s) was evacuated to dryness, redissolved in 0.2 ml of H_2O containing 2 μCi of ^{3}H-cAMP, then chromatographed on paper in MAA. The region of the chromatogram containing ^{3}H-cAMP was rinsed with toluene and eluted with H_2O. Aliquots of this eluate were chromatographed in a variety of systems. To test the effect of PDE on this ^{14}C-fraction, part of the above mentioned eluate was combined with an additional 0.3 μCi of ^{3}H-cAMP and divided into equal portions. PDE (20 μg) was added to one portion, and it was incubated for 5 hr at 30 C. After boiling for 3 min, both portions were chromatographed separately by cellulose TLC in MAA. One-cm zones were analyzed for ^{3}H and ^{14}C. To determine the ability of the unidentified ^{14}C-compound(s) to react in the binding assay, an additional 10-ml portion from the Dowex 50 eluate was evacuated to dryness, redissolved in 0.2 ml of H_2O containing 10 pmoles of ^{3}H-cAMP, and subjected to cellulose TLC in MAA. One-half-cm zones were scraped off and eluted with water. The eluates were evacuated and each was redissolved in 0.5 ml of H_2O. Twenty-five-μl aliquots from each were spotted on Milli-

pore filters, and the filters were analyzed for ^3H and ^{14}C. Fifty-μl aliquots of each 0.5-ml fraction were tested for binding assay activity at pH 4 and pH 4.5 by procedures described below.

Preparation of Tissues for Binding and Kinase Assays. Lettuce seeds (*Lactuca sativa* L., cv. Grand Rapids) were washed in 10% (v/v) Clorox for 10 min and then rinsed with H_2O until no odor could be detected. The seeds were germinated by aeration with forced air in a 50 μg/ml chloramphenicol solution at 24 C for 14 hr before use. Oat seeds (*Avena sativa* L., cv. Park) were also surface-sterilized in 10% Clorox, then dark-grown in Pyrex trays containing sterilized vermiculite. Coleoptiles with enclosed first leaves were harvested at 5 days. Potato tuber discs (*Solanum tuberosum* L.) approximately 8 $\times$ 1 mm were prepared by aeration in 50 μg/ml chloramphenicol at 24 C for 24 hr. Cell suspension cultures of tobacco (*Nicotiana tabacum* L.), grown in modified (20) medium of Linsmaier and Skoog (17), were subcultured onto the medium of Linsmaier and Skoog (17). Following 2 months of growth under continuous fluorescent light, the resulting callus was harvested under sterile conditions. Fresh rat and mouse livers were obtained from the College of Veterinary Medicine, Colorado State University.

Tissues used in the binding or kinase activation assay were ground in ice-cold 0.5 N $HClO_4$ containing either 10 or 100 pmoles of ^3H-cAMP in a Waring Blendor for 3 min, followed by homogenization with the Polytron for 2 min. (Only the Polytron was used for the mouse and rat livers.) Each homogenate was filtered through cheesecloth or was aspirated through Whatman No. 1 filter paper, and the filtrates were centrifuged. The supernatants were neutralized with KOH, chilled, centrifuged to remove $KClO_4$, freeze-dried, and redissolved in 3 to 12 ml of H_2O, depending on the amount of tissue used. The resulting solutions were purified by column chromatography using PVP followed by neutral alumina and Dowex 50. All columns were eluted with H_2O. Recovery estimates were made by measuring the ^3H-cAMP marker present in aliquots of the fractions collected from each column, and they ranged between 36 and 42% after the Dowex 50 step. The ^3H-cAMP-containing fractions from the Dowex column were pooled and used for routine cAMP assays.

Preparation of Protein Kinase. Protein kinase was prepared from 75 g of rabbit skeletal muscle (Pel Freeze Biologicals, Inc.) according to procedures similar to those described by Kuo and Greengard (16). Two major kinase peaks were eluted step-wise from the DEAE-cellulose column with consecutive 100-ml portions of 50 mM and 0.3 M potassium phosphate buffer, pH 7.0 and 1 mM Na_2EDTA. Peak I from the column was concentrated by ultrafiltration to 20 ml, while peak II was reduced to 4 ml; each was dialyzed overnight against 5 mM phosphate buffer, pH 7. One-ml portions of each dialyzed enzyme solution were frozen as stock solutions for later use.

Binding Assay. Protein kinase from peak I of the DEAE column described above was utilized in a modified Gilman binding assay (10). Standards of cAMP were prepared so that 50 μl of each solution contained from 1 to 32 pmoles of cAMP. Each assay tube contained 25 μl of 1% (w/v) BSA, 25 μl of binding protein (4-fold dilution of stock) 25 μl of 200 mM sodium acetate buffer (pH 4 or pH 4.5), 20 μl (1 pmole) of ^3H-cAMP, 20 μl of PDE (10 or 20 μg) or H_2O and 50 μl of standard cAMP, H_2O, or sample. Sample aliquots which contained significant amounts of ^3H-cAMP marker received proportionately less ^3H-cAMP for assay. The reaction was started by the addition of 75 μl of an equal volume mixture of acetate buffer, BSA, and binding protein. The assay tubes were kept on ice for 90 min, after which 2 ml of 20 mM potassium phosphate buffer, pH 6.5, were added, and the mixture was filtered through 22 mm, 0.45-μm HAWP Millipore filters. The filters were washed with 10 ml of the phosphate buffer and dried before ^3H analysis.

Kinase Assay. Peak II of the DEAE column was used for the kinase activation assay. The method used was essentially that of Kuo and Greengard (16) with minor changes. The assay tubes contained 100 μl of 0.1 M sodium acetate buffer (pH 6), 20 μl of 0.1 M $MgCl_2$, 4 μl (40 μg) of f1 calf thymus histone, 10 μl of protein kinase (undiluted stock), 50 μl of cAMP standard, H_2O, or sample, 20 μl of PDE (10 μg) or H_2O, and 10 μl of [γ-^{32}P]ATP (1 nmole). The reaction was started by adding the labeled ATP. The tubes were incubated at 33 C for 10 min. The reaction was stopped by placing the tubes in an ice water bath and adding 5 ml of ice-cold 25% (w/v) trichloroacetic acid to each. These mixtures were filtered through 22 mm, 0.45 μm, HAWP Millipore filters. Each filter was washed with 15 ml of 0.2 N $HClO_4$ and dried prior to ^{32}P analysis.

PDE Treatment of Binding or Kinase Activation Assay Samples. Purified extracts of various tissues were assayed with either assay before and after treatment with PDE. To obtain PDE-treated samples, 20 μl of PDE (10 or 20 μg in 20 mM ammonium acetate, pH 6.5) were added to 50 μl of purified extract and incubated at 30 C overnight. These samples were then boiled for 3 min before assaying. The effectiveness of the PDE was routinely checked by its effect on cAMP standards.

RESULTS

^{14}C-labeled Lettuce Seeds. Measurements of the absorption of labeled adenosine as a function of time yielded a smooth curve with 25% absorbed at 1 hr, 45% at 2 hr, 63% at 3 hr, 81% at 4 hr, and 96% at 5.5 hr. Figures 1 through 4 show the relative distribution of ^{14}C-labeled compounds and ^3H-cAMP after each of four purification steps performed in series on the lettuce seed extract. No distinct ^{14}C peak appeared with the ^3H-cAMP in any of these chromatographic steps. The Dowex 50 and electrophoresis separations did produce a ^{14}C peak just prior to the position of the ^3H-cAMP. The final separation with paper chromatography in EAA resulted in just 3 cpm of ^{14}C which migrated with the ^3H-cAMP. Further purification of the cAMP fraction was therefore prohibited. Table I summarizes the recovery of ^3H-cAMP marker and comigrating ^{14}C through each purification step.

The specific radioactivity of ATP in these extracts reached 6.9 $\pm$ 0.71 mCi/mmole after 5.5 hr of incubation in ^{14}C-adenosine. The concentration of ATP in the tissues at this time was 15 $\pm$ 1 μM. Using the specific radioactivity of ATP as an approximation of the specific radioactivity of cAMP, a maximum level of cAMP was calculated from the ^{14}C migrating with ^3H-cAMP in the final

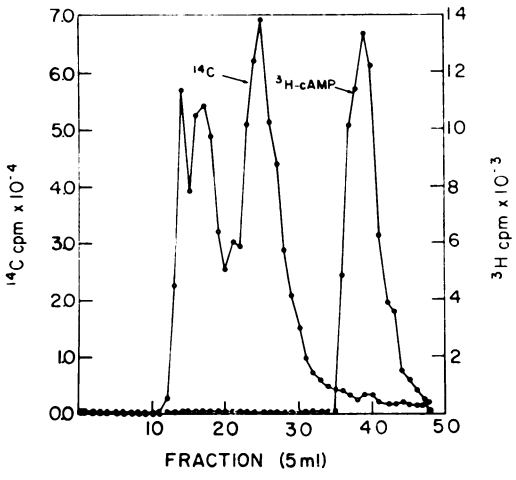

FIG. 1. Fractionation of neutralized $HClO_4$ extract of ^{14}C-adenosine-fed germinated lettuce seeds (half-sample from 20 g of seeds) containing ^3H-cAMP marker on a neutral alumina column with H_2O as the eluant.

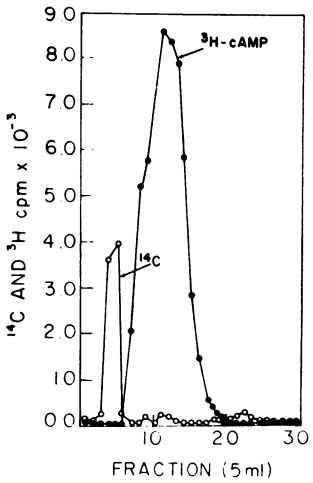

FIG. 2. Profile of ^{14}C compared to ^{3}H-cAMP marker of fractions 36–43 from alumina column (Fig. 1) fractionated on Dowex 50 column with H_2O.

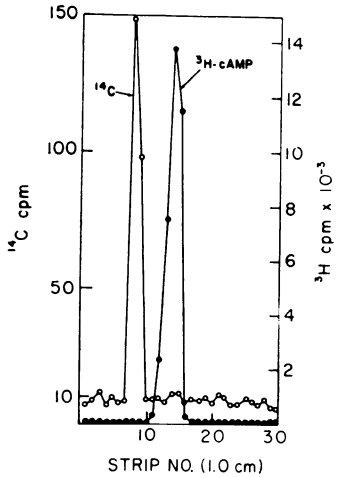

FIG. 3. Distribution of ^{14}C and ^{3}H-cAMP marker from fractions 7–16 off Dowex column (Fig. 2) after separation by paper electrophoresis. (Whatman No. 3MM paper run at 500 v for 5.5 hr).

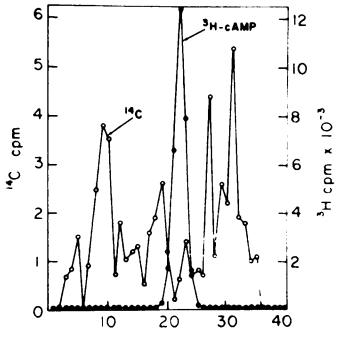

FIG. 4. Distribution of ^{14}C and ^{3}H-cAMP marker from strips 12–15 of electropherogram (Fig. 3) on a paper chromatogram developed in EAA. Spillover of ^{3}H (<0.004%) into the ^{14}C channel was subtracted from ^{14}C cpm. All ^{14}C values represent the mean of seven measurements of 40 min each.

purification step to be 0.37 pmole/g of unimbibed tissues (corrected for ^{3}H-cAMP losses).

Assay of Plant Tissues with Binding and Kinase Activation Assays. Standard curves for the binding and kinase activation assays are shown in Figure 5, a and b. The abilities of these assays to respond to 2′:3′-cAMP were examined, and the results are shown on the standard curves. The binding assay was not responsive to 2′:3′-cAMP at levels as high as 500 pmoles per tube (3.03 μM). The kinase activation assay did give a response at 1,000 pmoles/tube equivalent to less than 2 pmoles of cAMP, but this might have resulted from cAMP contamination of the 2′:3′-cAMP. The results of the assay of a variety of plant extracts after purification on PVP, alumina, and Dowex 50 columns with the binding and kinase activation assays are shown in Table II. Samples measured with the binding assay were also treated with 20 μg of PDE before measurement, and this resulted in 20 to 100% reduction in displacing activity measured. The presence of boiled PDE in the assay tubes causes a stimulation of the binding of the ^{3}H-cAMP to the protein kinase, and the results appear (because of more cpm bound) as if part or all of the displacing activity of the sample was destroyed by the PDE. The amount of original displacing activity removed in this way depends upon the amount of PDE used and the total displacing activity originally present. Unboiled PDE stimulates the binding even more than boiled enzyme, and centrifugation of the boiled PDE removes this stimulation. It is likely that the PDE acts in the same manner as the inhibitor protein described by Walsh *et al.* (31), which enhances the binding of ^{3}H-cAMP to the protein kinase. Brostrom and Kon (4) found that several proteins could produce the inhibitor effect. In further attempts to characterize the displacing activity of plant extracts with PDE, we reduced the amount of PDE used (from 20 μg to 10 μg) to minimize this effect.

The nonspecific nature of the binding assay on plant tissues is suggested by results of the kinase activation assay performed on the same extracts (Table II). The reasonable agreement between results obtained for mouse liver with both assays suggests that this nonspecificity of the binding assay is of less importance for animal tissues. The hypothesis that each of the means for the plant tissue kinase activation assay results in Table II was equal to zero was evaluated with the *t* test. The *t* values for the oat, tobacco, and lettuce allowed rejection of this hypothesis at the 95% confidence level, but the mean for the oat tissue was obtained from only two analyses.

Binding and Kinase Activation Assay of Individual Chromatographic Fractions. To study further nonspecificity in the binding assay and to evaluate the effectiveness of our extraction and purification techniques, individual fractions from alumina column chromatography of both lettuce seed and rat liver extracts were measured with the binding assay. Aliquots of all fractions were

Table I. *Summary of Purification of Half of ^{14}C-Adenosine-fed Lettuce Seed Extract*

Each subsequent purification was performed on the ^{3}H-cAMP containing region (see Figs. 1–4) of the previous chromatographic separation. All measurements of radioactivity were made at comparable counting efficiencies (31.0% for ^{14}C and 5.1% for ^{3}H) on Whatman No. 3MM paper.

Purification Step	^{3}H-cAMP	^{14}C	^{3}H/^{14}C	^{14}C Remaining
	cpm			%
1. Neutralized $HClO_4$ extract	165,000	15,140,000	0.01	100
2. Alumina column	70,300	22,600	3.11	0.149
3. Dowex 50 column	57,500	1,380	41.70	0.009
4. Paper electrophoresis	35,700	40	884	0.000
5. Descending paper chromatography in EAA	30,700	3	10,200	0.000

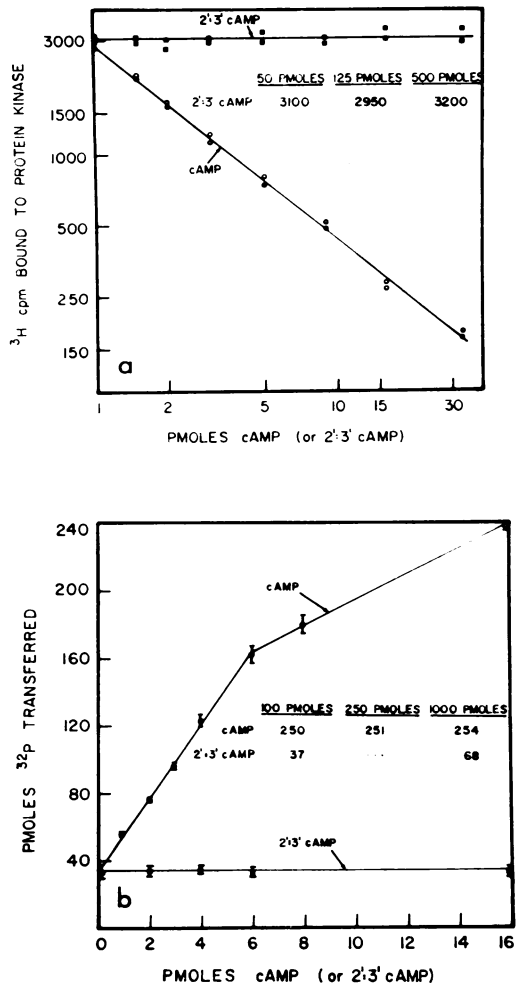

FIG. 5. a: Standard curve for cAMP and 2':3'-cAMP obtained with the binding assay. Ordinate shows the ^{3}H-cAMP cpm remaining bound to protein kinase as a function of pmoles of cAMP + ^{3}H-cAMP or 2':3'cAMP + ^{3}H-cAMP present in each reaction tube (165 μl). Approximately 20,000 cpm of ^{3}H-cAMP (1 pmole) were added to each tube. Insert shows displacing effects of 50, 125, and 500 pmoles of 2':3'cAMP as cpm remaining bound. Separate standard curves were run for all assays. b: Standard curve for kinase activation assay. Ordinate shows pmoles of ^{32}P transferred to f1 histone as a function of pmoles of cAMP or 2':3'cAMP present in the reaction tube (214 μl). Insert shows effects of up to 1000 pmoles of cAMP or 2':3'cAMP as pmoles ^{32}P transferred. Each point represents the mean ± SE of three replicates. Separate standard curves were run for all assays.

assayed: (*a*) alone, (*b*) after PDE treatment (10 μg), (*c*) with an additional 8 pmoles of exogenous cAMP, and (*d*) with 8 pmoles of cAMP followed by PDE treatment (10 μg). These results are shown in Figure 6, a and b. They indicate clearly the presence of nonspecific activity in the lettuce seed extract (Fig. 6a, fractions 2–5) and the absence of it in the rat liver extract (Fig. 6b), where the displacing activity matched the ^{3}H-cAMP marker. The addition of 8 pmoles of exogenous cAMP to aliquots of each fraction resulted in the measurement of approximately 8 additional pmoles in these aliquots, indicating the absence of any substances inhibitory to the detection of endogenous cAMP by this method in either the lettuce seed or rat liver extract. The effect of PDE on the displacing activity indicates the ability of the enzyme to degrade the added authentic cAMP in both lettuce seed and rat liver and the endogenous displacing activity in the rat liver, but inability to substantially remove the endogenous activity present in the lettuce seed extract. Thus the level of cAMP present in the lettuce seed extract was too low to be detected by this procedure.

Table II. *Summary of Estimations of cAMP Levels in Various Tissues by Use of Binding and Kinase Activation Assays*

Neutralized $HClO_4$ extracts were purified by PVP, alumina, and Dowex 50 column chromatography in series prior to assays. Exogenous cAMP added to samples was detected quantitatively. Doubling the amount of sample doubled the activity detected (except for the oat coleoptile extract which was not examined in this way). All values represent the mean ± SD of at least three replicates (except for oats in which there were only two anlayses) and are corrected for ^{3}H-cAMP losses.

Tissue	Amount of Tissue Used	Binding Assay	Kinase Activation Assay
	g fresh wt	*pmole cAMP equivalents/g*	
Mouse (liver)	3.2	471.2 ± 44.9	561.2 ± 44.7
Oat (coleoptiles and first leaves)	20.0	46.4 ± 1.9	1.9 ± 0.0
Lettuce (germinated seeds)	20.0[1]	2.2 ± 0.9	2.6 ± 0.7
Potato (aerated tuber discs)	20.0	30.4 ± 2.5	1.7 ± 1.3
Tobacco (callus culture)	5.5	68.5 ± 21.5	6.1 ± 4.2

[1] Unimbibed wt.

In the case of the lettuce seed extract, each of the fractions eluted from the alumina column was also analyzed with the kinase activation assay. Most of these fractions proved inhibitory to this assay, so fractions 5 to 7 (Fig. 6a) were combined and further purified on a Dowex 50 column. Figure 7 presents the results of an attempt to detect cAMP in the individual fractions of the Dowex 50 column with the kinase activation assay. Authentic cAMP added to aliquots of each fraction was quantitatively detected in almost every case, eliminating the possibility of inhibitors impeding the measurement of endogenous cAMP. There was a broad region of kinase stimulation which peaked in the same position as did the ^{3}H-cAMP internal standard. PDE apparently destroyed some of this activity (fractions 4–9). Failure of the PDE to destroy all of the exogenous cAMP could indicate that not all of the endogenous activity capable of being destroyed was eliminated by this enzyme. Even if all of the activity coeluting with ^{3}H-cAMP (fractions 5–8 of eluate curve) was in authentic cAMP, the maximum tissue concentration could have been only 2.8 pmoles/g unimbibed weight.

Nonspecific Displacing Activity of Potato Tuber Extracts. Figure 8 shows the binding activity of individual fractions from a Dowex 50 column used to purify 1 kg of potato tuber extract. The extract had been purified by PVP and alumina column chromatography prior to the Dowex 50. There are two large peaks of displacing activity, one eluting before and one after the ^{3}H-cAMP internal standard. Eight pmoles of exogenous cAMP added to aliquots of each fraction were detectable in addition to the endogenous displacing activity present, indicating that there was no inhibition of displacing activity in the ^{3}H-cAMP region. Compounds in the shoulder of displacing activity, overlapping the region of the ^{3}H-cAMP marker (fractions 7–10), were purified further by paper chromatography in EAA solvent. Figure 9 shows the displacing activity from eluates of the EAA chromatogram strips. Again, 8 pmoles of exogenous cAMP were quantitatively detected in nearly every case (data not shown). It appears, from the distribution of displacing activity into two major peaks, that activity eluting with the ^{3}H-cAMP marker on the Dowex 50 column (Fig. 8) resulted from contamination from the two major peaks there, and was not likely due to cAMP. This is emphasized by the decrease in specific radioactivity of displacing activity under the ^{3}H-cAMP peak from 0.099 pmole/cpm on the Dowex 50 column (Fig. 8) to 0.008 pmole/cpm on the paper chromatogram (Fig.

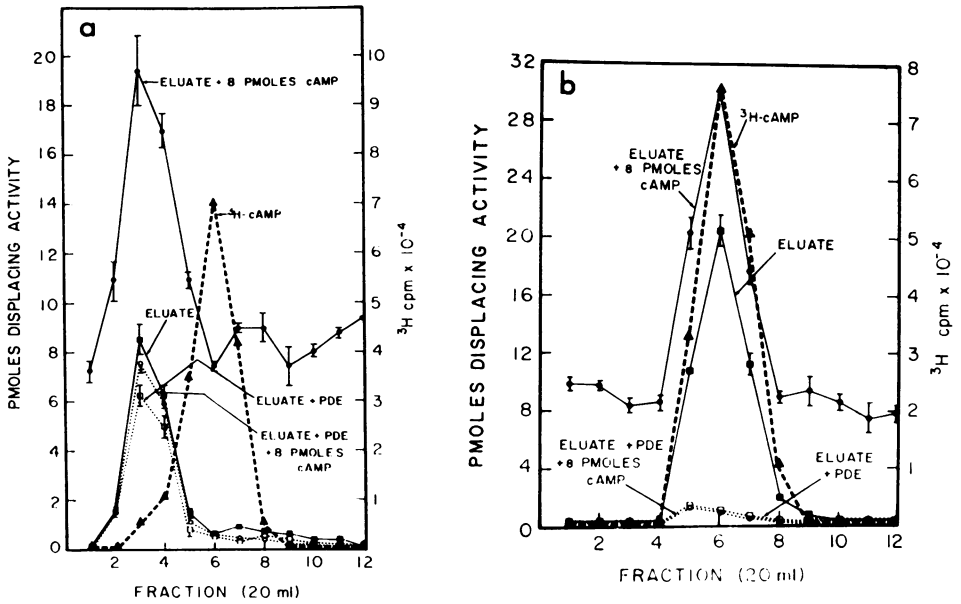

FIG. 6. a: Displacing activity of individual fractions from alumina column chromatography of neutralized extract of germinated lettuce seeds, 50 g unimbibed wt. Twenty ml fractions were reduced to 2 ml, and 50-μl aliquots of these tested. One pmole of displacing activity is equivalent to the number of ^{3}H-cAMP cpm displaced from the protein by 1 pmole of authentic cAMP. Ten pmoles of ^{3}H-cAMP were added to the grinding medium prior to homogenization. Each point represents the mean ± SE of three replicates. b: Displacing activity of individual fractions from alumina column chromatography of neutralized extract of rat liver, 7.1 g fresh wt. All experimental conditions and procedures were identical to those described for lettuce seed extract in 6a. Each point represents the mean ± SE of three replicates.

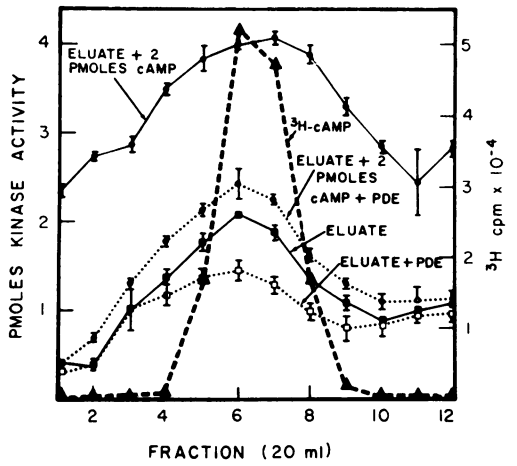

FIG. 7. Kinase activation of individual fractions from Dowex 50 column chromatography of fractions 5–7 (Fig. 6a) from alumina purification of neutralized extract of germinated lettuce seeds. Twenty-ml fractions were reduced to 2 ml, then 150-μl aliquots were reduced to 50 μl for analysis. One pmole of kinase activity is equivalent to the number of pmoles of ^{32}P transferred to f1 histone in the presence of 1 pmole of authentic cAMP. Each point represents the mean ± SE of three replicates.

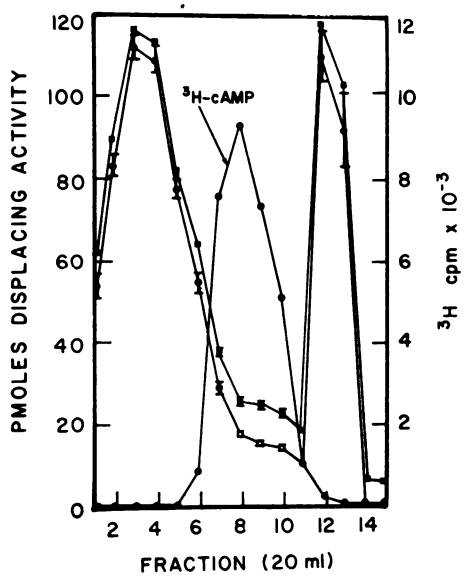

FIG. 8. Displacing activity of individual fractions of Dowex 50 fractionation of previously purified potato tuber (1 kg) extract. The 20-ml fractions were reduced to 2 ml, and 50-μl aliquots were tested with the binding assay. One pmole of displacing activity equals 1 pmole cAMP equivalent. Ten pmoles of ^{3}H-cAMP marker were added before homogenization. Values are given as the mean ± SE of three replicates, displacing activity (○), +8 pmoles of exogenous cAMP (■).

9). These data are included to illustrate the ability of the binding assay to detect activity which cochromatographs with cAMP during three successive purification attempts, but which can be resolved from cAMP by further purification. The concentration of cAMP displacing activity equivalents (24 pmoles/g fresh weight) in fractions migrating with ^{3}H-cAMP in Figure 8 proved similar to results for aerated potato tuber discs in Table II, where identical purification steps were employed.

Characterization of ^{14}C Compound(s) Similar to cAMP. Besides the detection of nonspecific activity with the binding assay in eluates from chromatographic systems designed to recover cAMP, we discovered, in the experiment in which ^{14}C-adenosine was used, that displacing activity was associated with a ^{14}C-fraction which originally eluted from the alumina column with ATP rather than with cAMP. Table III shows some chromatographic characteristics of this compound relative to cAMP. Although in some systems this ^{14}C fraction is very similar to cAMP, graphical data from chromatograms in which ^{14}C and ^{3}H-cAMP did not overlap

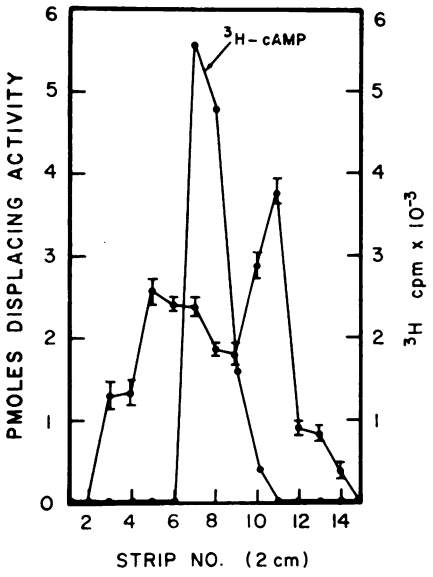

FIG. 9. Descending paper chromatography in EAA of fractions 7–10 off the Dowex 50 column (Fig. 8). Zones were eluted with H_2O, the eluate was brought to 2 ml, and 50-μl aliquots were tested in the binding assay. One pmole of displacing activity equals 1 pmole cAMP equivalent. Values are given as the mean ± SE of three replicates.

Table III. *Some Chromatographic Characteristics of ^{14}C Compound(s) Similar to cAMP from ^{14}C-Adenosine-fed Lettuce Seeds*

The ^{14}C fraction co-chromatographing with ^{3}H-cAMP on cellulose TLC in MAA was subjected to chromatography in the systems listed. Some systems separated the ^{14}C fraction into two subfractions.

Chromatography System	H-cAMP[3]	^{14}C	
		R_F values	
Cellulose-MAA	0.58	0.56	
Cellulose-EAA	0.58	0.46	
Cellulose-IAW	0.43	0.46[1]	0.33
Silica gel	0.67	0.67[1]	0.31
PEI-cellulose	0.70	0.87	
Cellulose-IBA	0.67	0.47[1]	0.75
Paper electrophoresis[2]	1.0	0.60[1]	1.43

[1] Minor peak.
[2] R_{cAMP}.

clearly showed that no part of this fraction could be authentic cAMP. Figure 10 indicates the displacing activity associated with this fraction and its remarkable chromatographic similarity to the ^{3}H-cAMP in the cellulose TLC-MAA system. There was more extensive displacing activity at pH 4 than at pH 4.5. Brostrom and Kon (4) showed that the nonspecific effects of various nucleotides and salts on the binding assay could be reduced by assaying at pH 4.5 instead of pH 4. The effect of the ^{14}C fraction (zones 12–15) on the binding assay is likely to be caused by the dissociation of the ^{3}H-cAMP rather than by competitive binding, since none of the ^{14}C from that region bound to the protein. Trace amounts of ^{14}C from the ATP region (zones 1–4) did bind to the protein, although no displacing activity was associated with this region. No detectable part of the ^{14}C fraction migrating with ^{3}H-cAMP in MAA was degraded by PDE to AMP (Fig. 11), while ^{3}H-cAMP present in the enzyme reaction mixture was over 90% degraded to AMP. This fraction inhibited rather than stimulated protein kinase activity.

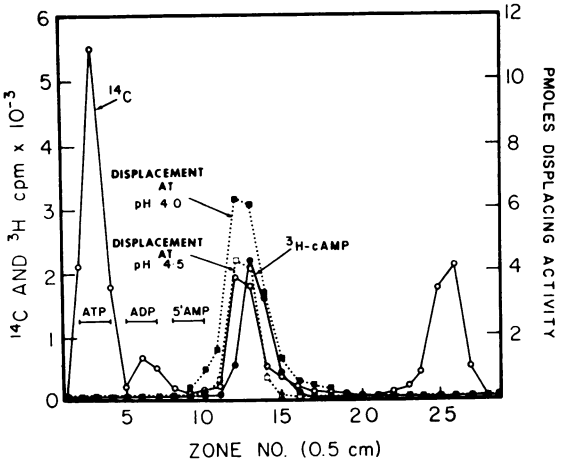

FIG. 10. Cellulose TLC in MAA of partially purified extract from ^{14}C-fed lettuce seeds. The extract was previously eluted from alumina with 0.2 M Na_2HPO_4, then this eluate was fractionated on Dowex 50 with H_2O prior to TLC in MAA. Zones were tested for displacing activity at pH 4 and pH 4.5. One pmole of displacing activity equals 1 pmole cAMP equivalent at the given pH. Distributions of ^{14}C and ^{3}H-cAMP marker are also shown.

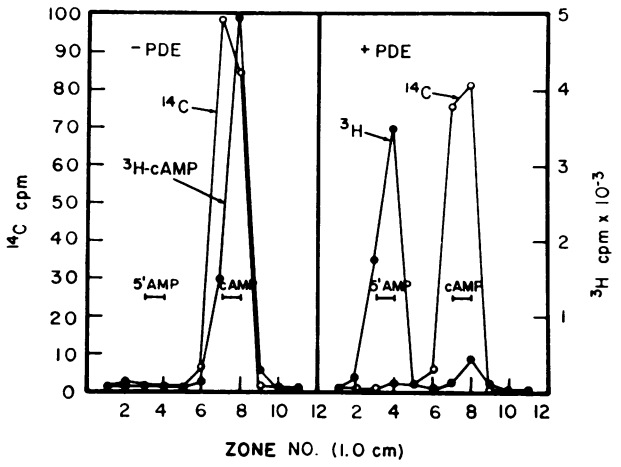

FIG. 11. Effect of PDE on ^{14}C compound(s) comigrating with ^{3}H-cAMP on cellulose TLC in MAA. Right graph: PDE-treated; left graph: not treated.

DISCUSSION

Isotopic Labeling. Our estimation of the level of cAMP in lettuce seeds by the adenosine labeling experiment (0.37 pmole/g unimbibed weight) assumed that cAMP reached the same specific radioactivity as ATP, that the 3 cpm of ^{14}C associated with the ^{3}H-cAMP in the last purification step represented an amount of radioactivity significantly greater than background, and that all 3 cpm were in cAMP. The progressive enrichment of the ^{3}H-cAMP fraction (Table I) places doubt on the authenticity of this putative ^{14}C-cAMP. It appears that if any cAMP was synthesized by the tissues, the endogenous level was below the reliable detection limit of this method. We feel that the use of relatively high amounts of labeled precursor (100 μCi), nearly total absorption by the tissues (>95%), and efficient purification methods (over 99% labeled contaminants removed by the first step) capable of handling relatively large amounts of tissue, brought this technique close to its maximum level of sensitivity. However, we emphasize that any cAMP not in isotopic equilibrium with the ^{14}C-ATP would result in an underestimate of its true endogenous level.

To demonstrate the presence of cAMP in plant tissues by this method, the putative ^{14}C-cAMP fraction should be brought to constant specific radioactivity with the ^{3}H-cAMP marker (*i.e.* ^{3}H/^{14}C remains constant through subsequent purifications), and a kinetic analysis of the effect of PDE on the ^{14}C fraction containing ^{3}H-cAMP should be performed such that the rate of degradation of the putative ^{14}C-cAMP is compared to that of the authentic ^{3}H-cAMP.

Binding Assay. The effectiveness of our purification procedures in removing ^{14}C-labeled contaminants is illustrated in Table I, which shows that the alumina and Dowex 50 columns removed over 99.99% of such contaminants. We therefore feel that the use of these columns (preceded by chromatography on a PVP column in some cases) to purify plant extracts for use in the binding and kinase assays removed a large portion of possible interfering compounds. Our studies indicate that the use of the binding assay to measure cAMP in higher plants may not be reliable because of interfering substances present even in highly purified preparations of plant tissues. The radioimmunoassay, by virtue of its similarity (in principle) to the binding assay, falls suspect as well. The detection of displacing activity in fractions of a purified plant extract chromatographically similar but not identical to cAMP (Fig. 10) illustrates the potentially deceptive nature of this assay. The ability of PDE to enhance the binding of ^{3}H-cAMP to the binding protein complicates the measurement of the effect of PDE on the displacing activity of fractions suspected to contain cAMP.

Luciferase Assay. We attempted repeatedly to measure cAMP in various plants with the luciferase assay (8) but usually did not obtain positive results, in spite of an earlier preliminary result (22). We also found it difficult to reproduce data consistently from this assay because of inadequate sensitivity resulting from contamination of the partially purified extracts and of the enzymes required in the assay by unidentified adenine nucleotides capable of producing high background values. Inhibitors of the luciferase and other enzymes used in the assay were encountered in plant extracts and in the paper or cellulose used for chromatography.

Kinase Activation Assay. The kinase activation assay is the most sensitive and reliable method we have used to measure endogenous cAMP in higher plants. Partially purified tissue extracts that produced high values in the binding assay gave much lower results in the kinase activation assay (Table II). Lettuce seed extracts purified only by alumina chromatography produced fractions that reacted positively in the binding assay but which were inhibitory in the kinase activation assay. Subsequent purification with Dowex 50 of the inhibitory fractions which coeluted with ^{3}H-cAMP from the alumina produced fractions which gave positive but low results in the kinase activation assay (Fig. 7). These results indicate a maximum endogenous cAMP level of 2.8 pmoles/g unimbibed weight. There is some nonspecific kinase stimulation activity associated with the purified lettuce seed extract (Fig. 7), and knowledge of the chromatographic behavior of activity in this assay appears important. The effect of PDE on these fractions (5–8, Fig. 7) supports the possibility that their activity is due to cAMP. The apparent removal of activity by PDE might be caused by its adsorption of active non-cAMP compounds rather than by its hydrolysis of cAMP. This phenomenon would become more likely as the molar ratio of PDE to active compounds becomes greater. Even if cAMP were present, its origin remains ambiguous, because the possibility of bacterial and human contamination cannot be ruled out in cases of such low cAMP levels. It is unlikely that cAMP was generated by either the alumina or the Dowex columns from ATP, since we failed to detect significant amounts of cAMP in the lettuce seed extract containing ^{14}C-ATP. In view of the demonstrated nonspecificity of the kinase activation assay, possible nonenzymatic removal of activity by PDE, and possible contamination, results from this assay in Table II are probably overestimated.

Any activity detected by either the binding assay or the kinase activation assay was probably not due to 2′:3′-cAMP, since both assays failed to respond to this nucleotide at concentrations as high as 0.47 μM (100 pmoles/assay tube). Yet, if tissue concentrations of this nucleotide were as high as approximately 1 μM, it could have caused some of the nonspecific activity, as many of the chromatographic systems used would not separate it from cAMP. Niles and Mount (21) attributed displacing activity from *Vicia faba* to 2′:3′-cAMP, although they cited no data showing that this nucleotide displaces cAMP from the binding protein.

Our results indicate that the endogenous levels of cAMP in the plants tested are considerably lower than levels typically reported for animal tissues as well as most measurements of cAMP in plants. Our results are in general agreement with those of Keates (14) and Ownby *et al.* (23), who used the adenosine-^{14}C labeling technique, and Amrhein (1), who employed the protein kinase activation assay. Both Keates and Ownby *et al.* purified a putative ^{14}C-cAMP fraction by successive steps that resulted in progressive enrichment of ^{3}H-cAMP marker until further purification was prohibited by low radioactivity of the fraction. Keates estimated the maximum cAMP level in barley aleurone layers to be about 2 pmoles/g fresh weight in the presence or absence of GA_3, while Ownby *et al.* estimated approximately 11 pmoles/g fresh weight in oat coleoptiles. Amrhein (1) ensured recovery of cAMP from tissue extracts by utilizing a ^{3}H-cAMP marker, and eliminated the possibility that inhibitory agents blocked the detection of cAMP in the kinase activation assay by adding authentic cAMP to aliquots of samples tested. He estimated cAMP levels in oat coleoptiles to be less than 25 pmoles/g fresh weight in one experiment and less than 8 pmoles/g fresh weight in another. He reported less than 2 pmoles/g fresh weight in tobacco pith, cultured *Catharanthus roseus* cells, and tomato seedlings. Considering this, a natural function for cAMP in these plants is questionable. Future efforts to demonstrate its presence should recognize the pitfalls of certain procedures. The possibilities that cAMP is present in only certain higher plant species, or that it appears at specific developmental stages exist, since the nucleotide is apparently present in some but not all bacteria (12), and the slime mold *Dictyostelium discoideum* produces cAMP during a specific developmental process (6). It remains possible that localization of cAMP in organelles or other subcellular sites places the functional level of this nucleotide near or below the reliable detection limit of present assay procedures when reasonable amounts of whole plant tissues are used.

Acknowledgments—We thank Patricia Fox for her very capable technical assistance, Charlotte Ownby, Department of Anatomy, for providing fresh mouse livers and Dr. Eugene Vigil, Department of Physiology and Biophysics, for providing fresh rat livers. We also wish to thank Dr. Murray Nabors, Department of Botany and Plant Pathology, for providing cell suspension cultures of tobacco, and Dr. John Hendrix for very helpful comments on the manuscript.

LITERATURE CITED

1. Amrhein, N. 1974. Evidence against the occurrence of adenosine-3′:5′-cyclic monophosphate in higher plants. Planta 118: 241–258.
2. Bachofen, S. 1973. Distribution of cyclic AMP in maize seedlings. Plant Sci. Lett. 1: 447–450.
3. Brewin, N. J. and D. H. Northcote. 1973. Variations in the amounts of 3′,5′-cyclic AMP in plant tissues. J. Exp. Bot. 24: 881–888.
4. Brostrom, C. O. and C. Kon. 1974. An improved protein binding assay for cyclic AMP. Anal. Biochem. 58: 459–468.
5. Brown, E. G. and R. P. Newton. 1973. Occurrence of adenosine 3′:5′-cyclic monophosphate in plant tissues. Phytochemistry 12: 2683–2685.
6. Chassy, B. M., L. L. Love, and M. I. Krichevsky. 1969. The acrasin activity of 3′,5′-cyclic nucleotides. Proc. Nat. Acad. Sci. U.S.A. 64: 296–303.
7. Drlica, K. A., J. M. Gardner, C. I. Kado, I. K. Vijay, and F. A. Troy. 1974. Cyclic adenosine 3′:5′-monophosphate levels in normal and transformed cells of higher plants. Biochem. Biophys. Res. Commun. 56: 753–759.
8. Ebadi, M. S., B. Weiss, and E. Costa. 1971. Microassay of adenosine-3′,5′-monophosphate (cyclic AMP) in brain and other tissues by the luciferin-luciferase system. J. Neurochem. 18: 183–192.
9. Giannattasio, M., E. Mandato, and V. Macchia. 1974. Content of 3′,5′ cyclic

AMP and cyclic AMP phosphodiesterase in dormant and activated tissues of Jerusalem artichoke tubers. Biochem. Biophys. Res. Commun. 57: 365–371.

10. GILMAN, A. G. 1972. Protein binding assays for cyclic nucleotides. *In:* P. G. Greengard, G. A. Robison, and R. Paoletti, eds. Advances in Cyclic Nucleotide Research, Vol. II. Raven Press, New York. pp. 9–24.
11. HALL, K. A. AND A. G. GALSKY. 1973. The action of cyclic-AMP on GA_3 controlled responses IV. Characteristics of the promotion of seed germination in *Lactuca sativa* variety 'Spartan Lake' by gibberellic acid and cyclic 3′,5′-adenosine monophosphate. Plant Cell Physiol. 14: 565–571.
12. HYLEMAN, P. B. AND P. V. PHIBBS. 1974. Evidence against the presence of cyclic AMP and related enzymes in selected strains of *Bacteroides fragilis*. Biochem. Biophys. Res. Commun. 60: 88–95.
13. JANISTYN, B. AND H. DRUMM. 1972. Light-mediated changes of concentration of c-AMP in mustard seedlings. Naturwissenschaften 59: 218.
14. KEATES, R. A. B. 1973. Evidence that cyclic AMP does not mediate the action of gibberellic acid. Nature 244: 355–357.
15. KESSLER, B., AND R. LEVINSTEIN. 1974. Adenosine 3′,5′-cyclic monophosphate in higher plants: Assay, distribution, and age-dependency. Biochim. Biophys. Acta 343: 156–166.
16. KUO, J. F. AND P. GREENGARD. 1972. An assay method for cyclic AMP and cyclic GMP based on their abilities to activate cyclic AMP-dependent and cyclic GMP-dependent protein kinases. *In:* P. G. Greengard, G. A. Robison, and R. Paoletti, eds., Advances in Cyclic Nucleotide Research, Vol. II. Raven Press, New York. pp. 41–50.
17. LINSMAIER, E. M. AND F. SKOOG. 1965. Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. 18: 100–127.
18. Lobayashi, Y. and D. V. Maudsley. 1970. Practical aspects of double isotope counting. *In:* E. E. Bransome, Jr., ed., The Current Status of Liquid Scintillation Counting. Grune and Stratton, Inc., New York.
19. MILLER, J. J. AND A. G. GALSKY. 1974. Radioimmunological evidence for the presence of cyclic-AMP in *Hordeum* seeds. Phytochemistry 13: 1295–1296.
20. NABORS, M., A. DANIELS, L. NADOLNY, AND C. BROWN. 1975. Sodium chloride tolerant lines of tobacco cells. Plant Sci. Lett. 4: 155–159.
21. NILES, R. M. AND M. S. MOUNT. 1974. Failure to detect cyclic 3′,5′-adenosine monophosphate in healthy and crown gall tumorous tissues of *Vicia faba*. Plant Physiol. 54: 372–373.
22. OWNBY, J. D., C. W. ROSS, R. A. BRESSAN, AND J. L. KEY. 1973. Studies on the presence of cyclic AMP in plants. Plant Physiol. 51: S-14.
23. OWNBY, J. D., C. W. ROSS, AND J. L. KEY. 1975. Studies on the presence of adenosine cyclic 3′:5′-monophosphate in oat coleoptiles. Plant Physiol. 55: 346–351.
24. POLLARD, C. J. 1970. Influence of gibberellic acid on the incorporation of 8-^{14}C adenine into adenosine 3′,5′-cyclic phosphate in barley aleurone layers. Biochim. Biophys. Acta 201: 511–512.
25. PRADET, A., P. RAYMOND, AND A. NARAYANAN. 1972. Confirmation de la presence de l'AMP cyclique dans les semences de laitue, var. Reine de Mai. C. R. Acad. Sci. Paris 275: 1987–1988.
26. RAST, D., R. SKRIVANOVA, AND BACHOFEN. 1973. Replacement of light by dibutyryl-cAMP and cAMP in betacyanin synthesis. Phytochemistry 12: 2669–2672.
27. RAYMOND, P., A. NARAYANAN, AND A. PRADET. 1973. Evidence for the presence of 3′,5′-cyclic AMP in plant tissues. Biochem. Biophys. Res. Commun. 53: 1115–1121.
28. SALOMON, D. AND J. P. MASCARENHAS. 1971. Auxin-induced synthesis of cyclic 3′,5′-adenosine monophosphate. Life Sci. 10: 879–885.
29. SALOMON, D. AND J. P. MASCARENHAS. 1971. The effect of cyclic 3′,5′-adenosine monophosphate on abscission of coleus petioles. Z. Pflanzenphysiol. 65: 385–388.
30. TRUELSEN, T. A., E. DELAUGHE, AND R. VERBEEK-WYNDAELE. 1974. Cyclic AMP induced growth promotion in sunflower callus tissue. Arch. Int. de Physiologie et de Biochimie. 82: 190–214.
31. WALSH, D. A., C. D. ASHBY, C. GONZALEZ, D. CALKINS, E. H. FISCHER, AND E. G. KREBS. 1970. Purification and characterization of a protein inhibitor of adenosine 3′,5′-monophosphate-dependent protein kinases. J. Biol. Chem. 246: 1977–1985.
32. WELLBURN, A. R., J. P. ASHBY, AND F. A. M. WELLBURN. 1973. Occurrence and biosynthesis of adenosine 3′,5′-cyclic monophosphate in isolated *Avena* etioplasts. Biochim. Biophys. Acta 320: 363–371.