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Cyclic Adenosine 3':5'-Monophosphate in Moss Protonema

A COMPARISON OF ITS LEVELS BY PROTEIN KINASE AND GILMAN ASSAYS

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ABSTRACT

From the protonema of the moss *Funaria hygrometrica* (L.) Sibth, a factor indistinguishable from cyclic adenosine 3':5'-monophosphate (cAMP) has been isolated. The factor stimulated the activity of protein kinase from rabbit skeletal muscle and co-chromatographed with authentic cAMP in two solvent systems. Its ability to stimulate protein kinase activity was completely abolished by 3':5'-cyclic nucleotide phosphodiesterase, the rate of inactivation being similar to that of authentic cAMP. Based on these properties, this factor is identified as 3',5'-cAMP. Cyclic AMP could be readily removed from the cells and washing the cells with water reduced the endogenous level of cAMP by 2- to 3-fold. A comparison of cAMP levels by protein kinase and Gilman assays was made. The intracellular levels determined by protein kinase assay were about 7-fold lower than the values obtained by Gilman assay. This discrepancy was due to the presence of unidentified compounds which were completely degraded by 3':5'-cyclic nucleotide phosphodiesterase. Although these displaced labeled cAMP in the Gilman assay, they did not stimulate the protein kinase activity. The protonema may contain cyclic nucleotides other than cAMP; these will not be detected in the protein kinase assay due to the specificity of this reaction. The crude extracts were found to be unsuitable for assaying cAMP by either method.

3',5'-cAMP is now recognized as a versatile regulator of many cellular processes (12). It is present in most of the bacteria where its best known function is a reversal of the catabolic repression (23). Among the fungi, it is found in *Blastocladiella emersonii* (28), *Coprinus macrorhizus* (30), *Neurospora crassa* (26), *Saccharomyces fragilis* (29) and cellular slime molds (10). In the slime mold *Dictyostelium discoideum*, cAMP has a morphogenetic effect and is involved in the aggregation of amoebae (10). Among Chl-containing organisms, *Chlamydomonas reinhardtii* (2) and *Euglena gracilis* (14) have been found to contain cAMP. It occurs in almost every animal tissue, including the mammalian body fluid (24). The red blood cells of some mammals and electric organ of the eel may constitute possible exceptions (24). The widespread distribution of cAMP in heterotrophic organisms has led many investigators to test for its possible occurrence and function in plants. The evidence for the presence of cAMP among higher plants usually deals with the isolation of a compound, which either competes with radioactive cAMP in isotopic dilution assays or is chromatographically similar to cAMP (see refs. 1 *and* 3). Enzymes that may function in cAMP metabolism have been reported in some cases (18). These results are, however, still controversial as the criteria applied for the identification of the factor as cAMP were not rigorous enough. As pointed out by Lin (18), the published accounts often lacked the crucial details of the methods employed.

We have recently reported that cAMP is involved in the differentiation of chloronema cells in the protonema of the moss *Funaria hygrometrica* (9). In order to elucidate the physiological significance of these results, we wanted to establish whether or not cAMP is present in this moss. The results presented here demonstrate that the *Funaria* protonema contains 3',5'-cAMP.

MATERIALS AND METHODS

Cultures of Moss Protonema. The experiments were carried out with the wild type cell line J-2 grown in axenic cell suspension cultures. These were comprised exclusively of chloronema cells which grew as short and multicellular filaments and their wet weight doubled after every 20 ± 2 hr. The method of isolating the cell line has been described earlier (11). The cell line was routinely maintained by diluting an aliquot of cells 25-fold with fresh low calcium medium (LCM). This medium (pH 5) contained/l, 50 mg $Ca(NO_3)_2 \cdot 4H_2O$, 250 mg KH_2PO_4, 250 mg KCl, 250 mg $MgSO_4 \cdot 7H_2O$, 813 mg KNO_3, 43 mg Ferric-EDTA complex, 10 ml Heller's micronutrient solution, and 10 g glucose. Heller's micronutrient solution was prepared by dissolving 100 mg $ZnSO_4 \cdot 4H_2O$, 10 mg $MnSO_4 \cdot 4H_2O$, 100 mg H_3BO_3, 3 mg $NiCl_2 \cdot 6H_2O$, 3 mg $AlCl_3$, 3 mg $CuSO_4 \cdot 5H_2O$ and 1 mg Kl in 1 liter of glass-distilled H_2O.

For cAMP extraction, the cells were inoculated at an initial cell density of 0.5 g/l of LCM[1] and the cultures were kept in continuous illumination (270 ft-c) at 26 ± 2 C. Before harvesting, cells were examined using the phase contrast microscope. Aliquots were also plated on L. Broth agar (10 g bacto tryptone, 5 g yeast extract, 5 g NaCl, 1 g glucose, and water to make 1 liter medium, final pH was adjusted to 6.7 with NaOH) to check for microbial contamination if any.

Isolation of PK-stimulating Factor from Protonema. Cells grown aseptically in LCM were harvested by filtering under vacuum through several layers of a nylon cloth, which was supported on a Pyrex 47-mm filter holder. The cells were frozen in liquid N_2 and powdered in a precooled mortar. The powdered cells were transferred to 200 ml of ice-cold 10% trichloroacetic acid containing a known amount of ^{3}H-cAMP (20.8 Ci/mmol) as internal standard and homogenized for 10 min in a Teflon homogenizer. The homogenate was centrifuged for 10 min at 19,000*g* at 0 C. The supernatant was decanted to a 1-liter separatory funnel and extracted with cold water-saturated diethyl ether until the pH of aqueous phase was above 4. The aqueous phase was then heated at 80 C to remove the residual ether, any precipitate formed at this step was removed by centrifugation. Purified Norit A (2 mg/ml) was added to the supernatant. The charcoal was recovered by filtration through two

[1] Abbreviations: LCM: low calcium medium; PK: 3':5'-cAMP-dependent protein kinase; PDE: 3':5'-cyclic nucleotide phosphodiesterase; cyclic GMP: cyclic guanosine 3':5'-monophosphate; cyclic IMP: cyclic inosine 3':5'-monophosphate.

layers of Whatman No. 1 filter paper, and was eluted with 2% NH_3 in 50% ethanol. The eluate was lyophilized, dissolved in 2 ml H_2O, and chromatographed on Bio-Rad Aminex MS cation exchange resin (packed volume 2 ml). The column was eluted with distilled H_2O and 2-ml fractions were collected. The position of cAMP was deduced from that of added ^{3}H-cAMP. The cAMP-containing fractions were pooled and treated with $ZnSO_4$-$Ba(OH)_2$ solution (15). The supernatant was lyophilized and further chromatographed on thin layer plates of Silica Gel G. Care was taken to ensure that cAMP was not formed nonenzymically from ATP. The chromatography on Aminex resin separated essentially all of the ATP from ^{3}H-cAMP and $ZnSO_4$ was always added before the addition of $Ba(OH)_2$ (4).

Chromatographic Solvents Used. The following solvent systems were employed: solvent A: isobutyric acid-ammonia-water, 66:1:33, v/v; solvent B: 1-butanol-methanol-ethyl acetate-ammonia, 7:3:4:4, v/v; solvent C: isopropyl alcohol-ammonia-water, 14:3:3, v/v.

Synthesis of γ-^{32}P-ATP. γ-^{32}P-ATP was prepared according to the procedure of Post and Sen (22) and purified over DEAE-Sephadex A-25 (25). Labeled ATP was diluted to 25 ml and loaded on a 2-ml column of DEAE-Sephadex. The column was eluted successively with 25 ml of 0.01 N, 10 ml of 0.1 N, 20 ml of 0.23 N, and 20 ml of 0.4 N NH_4HCO_3 in a stepwise manner. The first 10 ml of eluate with 0.4 N NH_4HCO_3 (containing most of the γ-^{32}P-ATP) was collected and desalted by adsorption to 200 mg of purified Norit A. The charcoal was eluted with 2% NH_3 in 50% ethanol, the eluate was lyophilized, and ATP was dissolved in glass-distilled H_2O. The radiopurity of each batch of ATP was established by chromatography on Whatman No. 1 paper in solvent A. The preparations invariably gave a single radioactive spot which chromatographed with authentic ATP.

The charcoal was purified before use by washing it successively with H_2O, absolute diethyl ether, 2% NH_3 in 50% ethanol, H_2O, 6 N HCl and H_2O, and finally dried.

Protein Kinase Assay. Protein kinase was partially purified from rabbit skeletal muscle (31) and the amount of enzyme to be added for an assay was predetermined for each enzyme preparation to ensure that it was not limiting up to 50 pmol of cAMP. In the initial experiments, assays were done following the procedure of Wastila *et al.* (31), while in latter ones, cAMP was determined as described by Kuo and Greengard (16) with the following modifications. The reaction volume was 200 μl instead of 186 μl. Under our standard assay conditions, each tube contained 0.05 M sodium acetate buffer (pH 6), 2 μmol magnesium acetate, 40 μg histone mixture, cAMP or plant extract in 100 μl, and 25 μl of cAMP-dependent protein kinase. Tubes were kept in ice during the addition of the enzyme. The reaction was commenced by the addition of 1000 pmol γ-^{32}P-ATP and tubes were incubated for 5 min at 30 C. The reactions were terminated by adding 2 ml of ice-cold trichloroacetic acid-tungstate-sulfuric acid mixture and processed (16). The protein was finally collected by centrifugation, dissolved in 0.1 ml of 1 N NaOH, and suspended in 10 ml of Bray's scintillation fluid for counting. A standard curve with authentic cAMP was prepared for each experiment and a typical one obtained following the conditions described above is shown in Figure 2. All assays were performed in duplicate.

Gilman Assay. The isotope dilution assay as described by Gilman (7) was used. The amount of ^{3}H-cAMP added as internal standard to monitor the recovery was less than 1% of the labeled cAMP used in the assays and the former did not interfere with the measurement of cAMP equivalents. All determinations were carried out in triplicate.

Measurement of Radioactivity. Samples containing isotope were counted in a Packard Tri-Carb liquid scintillation spectrometer. The efficiency of ^{3}H counting was about 40%.

Estimation of Protein. The samples were precipitated with trichloroacetic acid and acid-insoluble protein was determined (19) using BSA as a standard.

Hydrolysis with Phosphodiesterase. The samples containing plant extract or authentic cAMP were incubated for 30 min at 30 C in 100 μl of 40 mM tris-HCl buffer (pH 7.4) with 2 μmol of $MgSO_4$ and 5 μg (388 milliunits/mg protein) of active or boiled beef heart 3':5'-cyclic nucleotide PDE. The reactions were terminated by immersing the tubes in a boiling water bath and cAMP equivalents were determined using both protein kinase and Gilman assays.

Chemicals. All nucleotides and beef heart 3',5'-cyclic nucleotide phosphodiesterase were obtained from Sigma Chemical Co. $^{32}PO_4^{3-}$ (carrier free) was purchased from Bhabha Atomic Research Centre, Bombay. 8-^{3}H-cAMP (20.8 Ci/mmol) was bought from New England Nuclear. Enzymes for preparing γ-^{32}P-ATP and the kit for Gilman assay were bought from Boehringer-Mannheim. Silica Gel G and all other solvents (analytical grade) were from B.D.H. and Sarabhai Merck, India. The solvents were distilled once before use.

RESULTS

PK-stimulating Factor in Moss Protonema and its Identity with cAMP. We decided to use the protein kinase assay for cAMP measurements because it is relatively specific and is also highly sensitive (17). The cells were not washed with water after harvest as this reduced the endogenous level of cAMP (results presented later). The trichloroacetic acid-soluble extract and charcoal eluate from chloronema cells did not stimulate the activity of cAMP-dependent protein kinase and were strongly inhibitory (Table I). PK-stimulating activity became detectable only after the charcoal eluate had been chromatographed on Bio-Rad Aminex resin and further purified by treatment with $ZnSO_4$-$Ba(OH)_2$ as described under "Materials and Methods." On the basis of the UV absorbance, the chromatography of the charcoal eluate on Aminex resin resulted in a 100-fold purification. About 1% of the material absorbing at 260 nm was present in the ^{3}H-cAMP-containing fractions. The factor was characterized in detail to establish if the PK-stimulating activity in the protonema was due to the presence of 3',5'-cAMP.

Chromatographic Behavior of the PK-stimulating Factor from Protonema. The dry material obtained after the $ZnSO_4$-

Table I. Purification of PK-stimulating Factor from Chloronema Cells

The chloronema cells grown for 6 days in 2.2 liters of LCM were harvested (yield 53 g), homogenized in 10% trichloroacetic acid having ^{3}H-cAMP (37,300 cpm) and centrifuged. The supernatant showed 28,350 cpm (76% of input) and was used in the subsequent purification. Trichloroacetic acid soluble extract refers to the aqueous phase after the removal of ether. At each stage of purification, ^{3}H-cAMP and stimulation of PK-activity (31) were determined. The values given below have been corrected for the loss of cAMP at each step. In this experiment the intracellular level of cAMP was 18 pmol/g wet weight.

Purification step	^{3}H-cAMP recovered	recovery	cAMP equivalents	Specific radioactivity
	cpm	%	pmol	
1. Trichloroacetic acid-soluble extract	28,350	76	activity inhibited	-
2. Charcoal eluate	22,200	59	activity inhibited	-
3. Aminex column eluate	12,200	33	not done	-
4. Supernatant after $ZnSO_4$ - $Ba(OH)_2$	7,890	21	210	37
5. 1st TLC, solvent B	6,933	19	156	44
6. 2nd TLC, solvent C	6,168	15	172	36

$Ba(OH)_2$ precipitation step was dissolved in distilled H_2O and chromatographed on a thin layer plate of silica gel in solvent B. After development, the chromatogram was divided into 10 equal portions, which were individually eluted with 50% ethanol. Eluates from different regions were lyophilized, dissolved in distilled H_2O, and PK-stimulating activity determined. The entire activity was restricted to a single spot (R_F 0.5) which coincided exactly with the position of ^{3}H-cAMP (Fig. 1a). This material (R_F 0.5–0.6) was eluted and rechromatographed in solvent C, which separates 2',3'-cAMP from 3',5'-cAMP. The PK-stimulating factor again moved as a single spot which cochromatographed with authentic ^{3}H-cAMP (Fig. 1b). The specific activity of PK-stimulating factor after the $ZnSO_4$-$Ba(OH)_2$ precipitation step was 40 ± 4 cpm/pmol cAMP and it remained more or less constant through two subsequent chromatographic steps in different solvent systems (Table I). These results show that stimulation of PK activity in the extract is entirely due to a substance similar to ^{3}H-cAMP. If the PK-stimulating activity was due to something not identical with ^{3}H-cAMP, then it should have separated from the internal standard on further purification by chromatography.

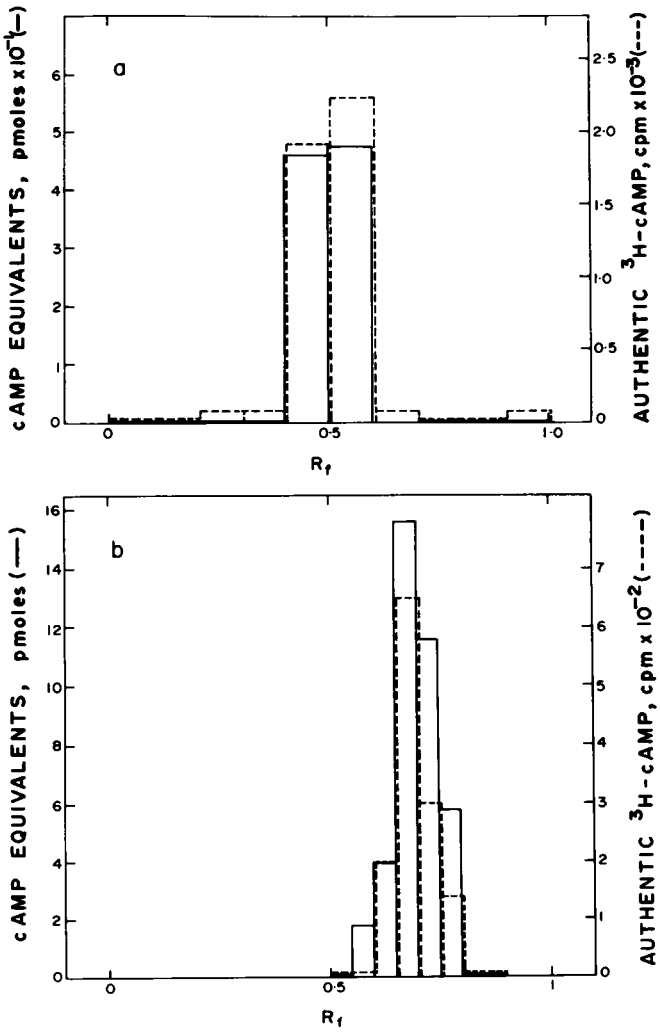

FIG. 1. Chromatography of PK-stimulating activity from protonema on thin layer plates of silica gel. ^{3}H-cAMP added as internal standard was equivalent to 0.25% of the total PK-stimulating activity of the sample. a: Factor from protonema after $ZnSO_4$-$Ba(OH)_2$ precipitation step was chromatographed in solvent B. b: Material between R_F 0.5 and 0.6 after first chromatography was eluted and rechromatographed in solvent C. The chromatogram was divided into 8 equal portions (R_F 0.5–0.9); ^{3}H-cAMP and PK-stimulating activity (31) in each were determined. On right axis, 18,000 cpm represent 1 pmol of cAMP.

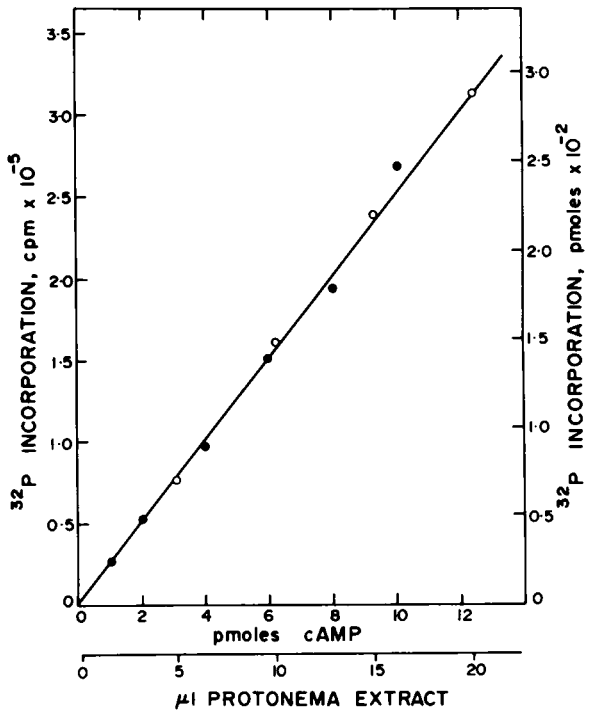

FIG. 2. Phosphorylation of histone as a function of increasing concentrations of protonema factor (○) and authentic cAMP (●). The factor was obtained by purifying the trichloroacetic acid-soluble extract on Aminex resin and TLC in solvent B and was assayed by the stimulation of PK activity (16). Values have been corrected for the cAMP-independent phosphorylation.

Activation of PK with Factor from Protonema. A linear relationship between the amount of factor and the activation of PK was obtained (Fig. 2). This result shows that the stimulation of PK depends on the concentration of factor from protonema in the same way as that of the authentic cAMP. If this factor and cAMP are identical, their effects on the stimulation of PK activity should also be additive. The data presented in Table II show that this is in fact the case. These experiments also rule out the possibility that the partially purified extracts contained materials inhibitory for the kinase action. Therefore, we have not underestimated the cAMP levels.

Kinetics of Inactivation of PK-stimulating Factor with PDE. The beef heart cyclic nucleotide PDE completely abolished the PK-stimulating activity of the factor from protonema. This inactivation can also be due to other enzymes present as contaminants (in the PDE), which can hydrolyze adenine derivatives (8). To rule out this possibility, the kinetic behavior of inactivation of PK-stimulating factor and of authentic cAMP by PDE was examined. The rates of inactivation of both were found to be the same (Fig. 3).

The results presented so far strongly suggest that the PK-stimulating activity in the purified extracts is due to cAMP.

Comparison of Endogenous Level of cAMP Equivalents by Protein Kinase and Gilman Assays. These results were followed up by measuring the cAMP level by Gilman assay which has been used very widely. This assay is more convenient for determining cAMP in a large number of samples routinely. We also wanted to know if two independent methods yielded comparable values. The levels of the material scored as cAMP by Gilman assay were observed to be consistently higher than those obtained by the PK assay. To resolve this discrepancy, cAMP equivalents were determined at each stage of purification of the

Table II. Effect of Adding Factor from Protonema on the Protein Kinase Stimulating Activity of Authentic cAMP

The PK-stimulating factor from protonema was purified as described in Fig. 2. Cyclic AMP was assayed as described by Kuo and Greengard (16). 1100 cpm incorporated in histone represent one pmol $^{32}PO_4$. 5 μl of protonema extract was found to contain 3.1 pmol of cAMP-equivalents. Cyclic AMP-independent incorporation of $^{32}PO_4$ has been substracted from all determinations.

Factor from protonema	Authentic cAMP	Total[1] cAMP	^{32}P incorporated measured	expected[2]
μl	pmol			
-	2	2	49	-
-	4	4	89	-
-	6	6	139	-
5	-	3.1	71	72
5	2	5.1	123	119
5	4	7.1	177	165
5	6	9.1	239	212
10	-	6.2	150	145
10	2	8.2	206	191
10	4	10.2	257	238

[1]Authentic cAMP + cAMP from protonema extract.

[2]The mean value for the pmoles of ^{32}P incorporated per pmole of cAMP was obtained from the three concentrations of cAMP given at the top of the table. The total ^{32}P incorporated (expected values) was then calculated from this mean value and the total amount of cAMP.

crude extract by both the procedures. The purification of cAMP achieved at various steps can be inferred from the specific radioactivity (cpm/pmol cAMP equivalents) of the ^{3}H-cAMP used as internal standard. An increase of specific radioactivity will indicate the removal of contaminating materials which displace the labeled cAMP in the Gilman assay. The material measured as cAMP at various steps was tested for its sensitivity to cyclic nucleotide PDE. These criteria could not be applied to crude extracts as these completely inhibited the activity of PDE. The chromatography on the Aminex resin resulted in the removal of these inhibitory compounds. At this stage, the material competing with labeled cAMP in the Gilman assay was completely degraded by PDE (Table III). In the protein kinase assays, the eluate from Aminex resin inhibited the PK activity unless precipitated with $ZnSO_4$-$Ba(OH)_2$. In the absence of the latter step, the PK-stimulating activity could be separated from inhibitory materials by chromatography on thin layer plates of silica gel in solvent B. The distribution of cAMP equivalents determined by Gilman and PK assays on the chromatogram is shown in Figure 4. The distribution of PK-stimulating factor coincided completely with that of ^{3}H-cAMP used as internal standard. This observation confirms the results described earlier (Fig. 1a). In the Gilman assay, on the contrary, cAMP-like material was present throughout the chromatogram. The major peak of the material displacing labeled cAMP did not coincide with the fraction containing ^{3}H-cAMP. A further analysis showed that PK-stimulating activity and ^{3}H-cAMP were distributed uniformly throughout the peak. The specific radioactivity of ^{3}H-cAMP in the trailing and leading halves of the peak (marked a and b in Fig. 4) was the same—69 and 70 cpm/pmol cAMP, respectively. The concentration of cAMP equivalents based on Gilman assay in fraction a was 14-fold higher than that determined by PK assay (Table III). However, all of the material measured by Gilman assay was completely degraded by PDE. In order to see the behavior of this material, the eluate from fraction a was rechromatographed on thin layer plate of silica gel in the solvent B. As shown in Figure 5, the PK-stimulating activity was confined to a single peak and the specific radioactivity of fractions c and d remained unaltered on rechromatography (Table III). The Gilman assay indicated that the material competing with labeled cAMP was distributed in at least three additional regions. After rechromatography, the recoveries of ^{3}H-cAMP and of PK-stimulating activity was 90% or more. On the other hand, only 55% of the material measured by Gilman assay was recovered. Fractions c and d showed only 14% of the material present initially in fraction a. The material displacing labeled cAMP in Gilman assay appears unstable. This could account for the appearance of additional peaks and for the low recovery upon chromatography. The ^{3}H-cAMP and PK-stimulating activity did not undergo any change during rechromatography.

In the crude extracts, Gilman assay indicated the presence of 202 pmol of cAMP equivalents/g wet weight. On further purification, this value decreased to about 63 pmol/g wet weight, which is still higher than the value of 30 pmol/g wet weight determined by PK assay (Table III). These results clearly demonstrate that most of the material which competes with ^{3}H-cAMP in the Gilman assay is in fact not identical with cAMP, and this assay is unsuitable even for the extensively purified plant extracts.

Based on PK assay, the two experiments discussed so far indicated the presence of 18 and 32 pmol of cAMP equivalents/g wet weight, respectively (Tables I and III). Later experiments showed that the intracellular level of cAMP was highest in the cells at late exponential phase of growth. These contained about 44 to 56 pmol/g wet weight (9).

Effect of Washing on the Endogenous Level of cAMP Equivalents. While estimating cyclic nucleotide levels in chloronema cells, it as observed that washing the cells with H_2O before extraction of cAMP greatly reduced its intracellular levels.

To study the effect of washing, cells grown for 5 days in LCM were harvested. Half of these were immediately processed for cAMP extraction (unwashed), while the remaining were washed with 500 ml of distilled H_2O while still on the nylon cloth and then processed for cAMP extraction (washed). The trichloroacetic acid-soluble extracts were purified on Aminex resin followed by TLC in solvent B and cAMP levels were determined using the PK assay (16). The unwashed cells contained 31 pmol cAMP/g wet weight (1.14 pmol/mg protein), and this was reduced to 13

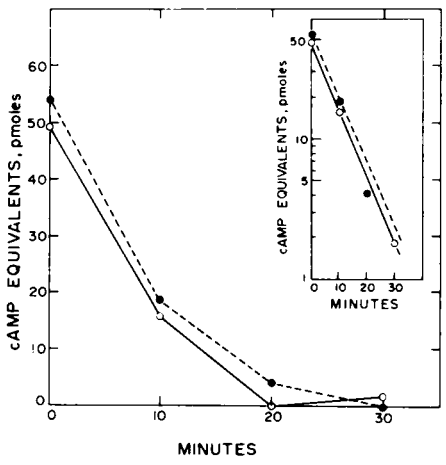

FIG. 3. Kinetics of inactivation of PK-stimulating factor from protonema (○——○) and authentic cAMP (●- - -●) by beef heart 3′:5′-cyclic nucleotide phosphodiesterase. Cyclic AMP and factor from protonema (about 60 pmol cAMP equivalents of each) were incubated in 120 μl of 40 mM tris-HCl buffer (pH 7.4) containing 2 mM $MgSO_4$ and 2 milliunits of PDE. The partially purified protonema factor was obtained as described in Figure 2. Aliquots of 20 μl each were removed at various intervals as indicated, kept in boiling water bath for 5 min, and then chilled in ice bath. The concentration of factor and cAMP were determined (31). Inset shows a semilogarithmic plot of these data.

Table III. A comparison of cAMP-Equivalents determined by Protein Kinase and Gilman-Assays at Various Stages of Purification

The chloronema cell grown for 3 days in 2 liters of LCM (yield 40 g) were homogenized in 6% trichloroacetic acid having 80,000 cpm of ^{3}H-cAMP. The trichloroacetic acid-soluble extract was lyophilized. The dry material was dissolved in distilled H_2O and chromatographed on Aminex resin. ^{3}H-cAMP containing fraction was chromatographed on thin layer plates of silica gel as described in Figures 4 and 5. Cyclic AMP equivalents were estimated by protein kinase (16) and Gilman-assays. Since 50% of the total sample obtained from fraction-a was chromatographed on 2nd TLC, the values given in table have been corrected for the total sample volume. In this experiment, the absorption on charcoal and precipitation by $ZnSO_4$ - $Ba(OH)_2$ were omitted to avoid the exposure of the extract to alkaline conditions.

Purification Step	Protein kinase-assay				Gilman-assay				Ratio of cAMP Equivalents Gilman-assay: PK-assay
	Total	PDE sensitive	Specific radioactivity	per g wet weight	Total	PDE sensitive	Specific radioactivity	per g wet weight	
	pmol cAMP-equivalents								
1. Trichloroacetic acid-soluble extract	activity inhibited	-	-	-	5800	activity inhibited	9.9	202	-
2. Aminex column eluate	activity inhibited	-	-	-	2144	2144	14.3	140	-
3. 1st TLC, solvent B									
fraction a	126	126	68.8		1776	1776	4.9		14.00
fraction b	208	208	70.9		669	669	21.5		3.40
fraction a+b	334	334	70.9	28.53	2445	2445	9.5	210	7.40
4. 2nd TLC of fraction a, solvent B									
fraction c	41	41	64.0		64	60	43.5		1.47
fraction d	72	72	63.4		180	168	27.2		2.35
fraction c+d	113	113	63.5	31.50	244	228	31.5	63	2.00

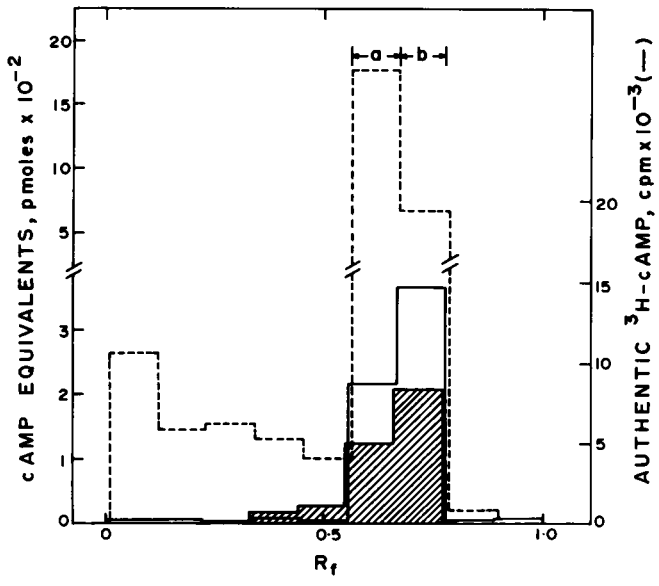

FIG. 4. Distribution of ^{3}H-cAMP, PK-stimulating activity and cAMP equivalents detected by Gilman assay on the thin layer chromatogram. Only the PDE-degradable material has been shown. The eluate from Aminex column containing 30,680 cpm of ^{3}H-cAMP was lyophilized. The dry material was dissolved in distilled water and chromatographed in solvent B. The chromatogram was divided into nine 2-cm wide portions and each was eluated with 50% ethanol. The cAMP equivalents were determined in each fraction ± phosphodiesterase by both the assays as described earlier. Shaded area: protein kinase assay; (—) Gilman assay.

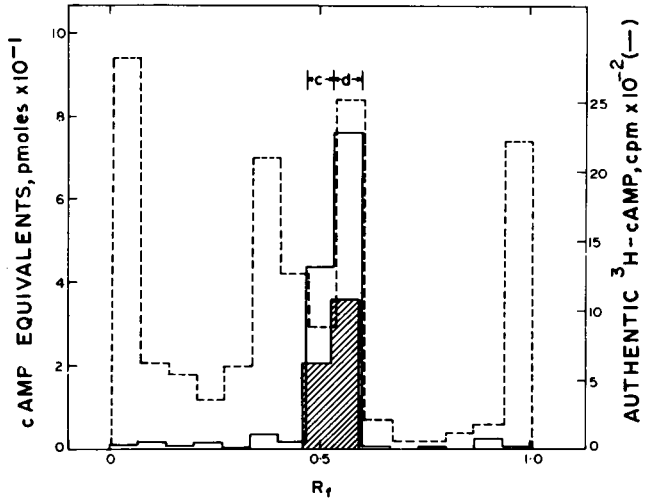

FIG. 5. Distribution of ^{3}H-cAMP, and cAMP equivalents detected by Gilman and protein kinase assays after rechromatography of fraction a (marked in Fig. 4) in solvent B. The chromatogram was divided into 15 1-cm wide portions. Other experimental conditions are the same as described in Figure 4. Shaded area: protein kinase assay; (----): Gilman assay. Only half of the eluate was rechromatographed.

pmol/g wet weight (0.53 pmol/mg protein) after washing.

In a separate experiment, effect of washing on the level of cAMP-like material was investigated. The trichloroacetic acid-soluble extracts from washed and unwashed cells were purified on Dowex 50 [H$^+$] resin and cAMP equivalents were determined by Gilman assay (7). The unwashed cells showed 361 pmol cAMP equivalents/g wet weight (21.33 pmol/mg protein), whereas the washed cells contained only 121 pmol cAMP equivalents/g wet weight (7.16 pmol/mg protein). These results show that both cyclic AMP and cAMP-like material are easily removable by washing the cells with H_2O.

DISCUSSION

There have been many attempts to demonstrate the presence of cAMP in higher plants. Despite many claims and counterclaims, its occurrence in this group of plants still remains extremely doubtful (1, 3, 18, 21). In extensive reinvestigations, no conclusive evidence for the occurrence of cAMP was found in several higher plants either by means of protein kinase assay or by the incorporation of adenine or adenosine (1, 3, 13, 21). There is also no conclusive evidence for the occurrence of either adenyl cyclase or of a phosphodiesterase specific for 3',5'-cyclic nucleotide in higher plants. Cyclic AMP and/or enzymes of its metabolism have so far been recorded only in the green alga *C.*

reinhardtii, fungi, and bacteria (2, 5, 6, 10, 23, 27, 28, 30). Cyclic AMP seems to play an important role in regulating the morphogenesis in bacteria and fungi (10, 23, 26). The situation in lower plants seems different from that of higher plants.

The results presented in this paper demonstrate that the *Funaria* protonema contains a factor which stimulates the activity of PK from rabbit skeletal muscle. This factor was shown to be identical with 3',5'-cAMP on the basis of co-chromatography and rates of inactivation by PDE. We argue that these results cannot be explained due to 3',5'-cyclic IMP, 3',5'-cyclic GMP, or other cyclic nucleotides which are also known to be active in the PK assay but only at a much higher concentration. In the solvent systems we used, these compounds moved to a R_F different from that of 3',5'-cAMP and all of the PK-stimulating activity was observed to be present in the region of 3',5'-cAMP. The presence of any significant amounts of purine and pyrimidine derivatives after the precipitation of extract with $ZnSO_4$-$Ba(OH)_2$ is ruled out, as this procedure removes most of these compounds (15). On the basis of these results, we conclude that *Funaria* protonema contains 3',5'-cyclic AMP. One would like to have a rigorous proof for the chemical identity of PK-stimulating factor and cAMP, but at present it is difficult to do so because of limitations in preparing this compound on a preparative scale. The intracellular levels of cAMP (pmol/mg protein) determined by PK assay in moss protenema agree closely with the values reported in a variety of animal tissues in the absence of stimulation by an exogenous hormone (24).

Once it was obvious that protonema contains cAMP, we wanted to use Gilman assay for routine measurements. This assay has been employed to measure cAMP even in crude extracts (7). Based on Gilman assay, the level of material scored as cAMP in protonema was at least seven times higher than that determined by using PK assay. It therefore became necessary to investigate the reason for high values obtained using the former assay. Since the Gilman assay is based on the displacement of labeled cAMP from the cAMP-binding protein by cAMP in the sample (7), any other substance lowering the binding of labeled cAMP will also be scored as cAMP. These drawbacks are not present in the PK assay. In this assay, the inhibitory compounds will decrease the stimulation of kinase activity and by including appropriate controls (assaying samples in the presence of a known amount of cAMP), cAMP, if present in the samples, can be fairly accurately estimated. To employ these assays meaningfully, the problem is to devise a purification procedure that eliminates the inhibitory and interfering materials. In moss protonema, the chromatography of the crude material on Aminex resin followed by TLC yielded preparations acceptable for PK assay. At this stage, all of the PK-stimulating activity was associated with ^{3}H-cAMP peak and no compounds inhibitory for kinase activity were present.

Measurement of cAMP by Gilman assay showed 7-fold more cAMP equivalents as compared to PK assay, after the first TLC. This was due to the presence of a material which was PDE-degradable, and a major part of it overlapped with ^{3}H-cAMP on a thin layer chromatogram. On further purification by chromatography, its amount was greatly reduced and it seemed to break down giving additional peaks. After the second TLC, Gilman assay still showed 2-fold more cAMP equivalents.

The chemical instability of the material which partly moved with ^{3}H-cAMP during first TLC is an interesting and a notable feature. While investigating the possible incorporation of ^{14}C-adenine into cAMP by *Avena* coleoptile sections, Amrhein (1) found that the peak of putative ^{14}C-cAMP was resolved into three separate peaks on chromatography on DEAE-Sephadex A-25. Most of the ^{14}C activity preceded the internal standard of ^{3}H-cAMP and was not present in cAMP. The inclusion of charcoal adsorption step followed by elution with NH_4OH in ethanol resulted in a considerable decrease of the ^{14}C material. It was suggested that the ^{14}C-metabolite was either unstable or had a higher affinity to charcoal (1). We would like to suggest that in moss protonema, the contaminant giving high values using the Gilman assay is perhaps similar to the unstable ^{14}C-metabolite of adenine. In both cases, the exposure to alkaline conditions seems to have led to a degradation of cAMP-like material. This line of argument seems to suggest the presence of additional cyclic nucleotides (because all of the material in moss was completely degraded by cyclic nucleotide PDE) which appear to be similar to cAMP but not identical with it. Washing of chloronema cells with water not only lowered the cAMP level (PK assay) but also of the material apparently scored as cAMP by Gilman assay.

We have ruled out the possibility of this material being 2',3'-cAMP. Employing Gilman assay, Niles and Mount (20) reported that 2',3-cAMP competed with 3',5'-^{3}H-cAMP, although they gave no data and cited no reference supporting it. Based on differential activities of PDE from beef heart and carrot tissues toward the two isomers of cAMP, they identified the competing material from *Vicia faba* internodes as 2',3'-cAMP (20). To find out if the cAMP-like material from protonema was 2',3'-cAMP, we have tested the latter's interference under our assay conditions. The results showed that at a low concentration (10 pmol) 2',3'-cAMP does not displace labeled cAMP. At very high levels, there was some competition, 10,000 pmol of 2',3'-cAMP were scored equivalent to 3 pmol of 3',5'-cAMP. Bressan *et al.* (3) have also reported recently that 2',3'-cAMP does not compete in Gilman assay. These results show that 2',3'-cAMP does not interfere in Gilman assay and therefore the compound identified in *V. faba* as 2',3'-cAMP (20) cannot have that structure.

In plants, compounds competing with labeled cAMP in binding assay seem to be fairly widely distributed and results very similar to that obtained in the present study have been recently described by Bressan *et al.* (3). Their work also demonstrates that even the rigorously purified preparations from several higher plants gave much higher values of cAMP equivalents by Gilman assay as compared to PK assay. In lettuce seeds, only a part of this material competing in binding assay was removed by PDE treatment (3), whereas in moss protonema, this material was completely degraded by PDE. These authors (3) have also pointed out a possible source of error arising due to increased binding of labeled cAMP to the binding protein in the presence of high amounts of PDE. Our results on the PDE sensitivity of cAMP-like material from protonema (Table III and Figs. 4 and 5) cannot be explained due to increased binding. We used a very low amount of PDE (5 μg/assay tube), and moreover, boiled PDE (5 μg) was also included in each assay tube while preparing the standard curve (Gilman assay).

Our observation that washing of cells leads to a decrease in cAMP content is significant. Cells in some bacteria and fungi release cAMP into the medium (10, 23). At this stage, it is not known if the chloronema cells also release cAMP into the medium. If the lowering of endogenous cAMP level by washing of cells turns out to be a general phenomenon, then in some of the investigations where cells were extensively washed before extraction, cAMP content may have been underestimated.

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