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BRIEF NOTES

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TRANSFORMATION OF TROPHIC *HARTMANNELLA CULBERTSONI* INTO VIABLE CYSTS BY CYCLIC 3′,5′-ADENOSINE MONOPHOSPHATE

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INTRODUCTION

Hartmannella culbertsoni, a free-living soil ameba, undergoes differentiation to be transformed into a double-walled cyst when exposed to a nonnutrient medium containing magnesium ions and taurine (Raizada and Krishna Murti, 1971). The transformation of the trophic form to mature cysts is usually preceded by a stage during induction when the ameba assumes a round and well-defined shape after the withdrawal of the pseudopodia. Evidence is adduced in this communication to show that cyclic 3′,5′-adenosine monophosphate (CAMP) can replace magnesium ions and taurine in the nonnutrient agar medium and that CAMP brings about a comparable degree of encystation of the ameba. The presence of magnesium ions and taurine has also been shown to result in a three- to fourfold increase in the rate of synthesis of CAMP by the ameba.

MATERIALS AND METHODS

Hartmannella culbertsoni (Singh and Das, 1970) was kindly provided by Dr. B. N. Singh of this Institute. The ameba was grown axenically in a medium consisting of 1% w/v proteose-peptone, 1% w/v tryptone and 0.5% w/v NaCl, pH 6.8. The amebae were harvested and processed for the differentiation studies as described elsewhere (Raizada and Krishna Murti, 1971).

Transformation to Cysts

Nonnutrient agar plates (10-cm diameter Petri dishes) containing 15 mM $MgCl_2$ and 20 mM taurine or CAMP with or without cycloheximide or actinomycin D were prepared. 1 ml of an aqueous suspension containing 10^4 amebae harvested from 6-day-old axenic cultures was spread on the plates and incubated at 26 ± 1°C. Loopfuls taken from the surface of the plates were examined at intervals for the appearance of cysts. Using triplicate samples, the number of cysts and trophic amebae at any given time was computed with the aid of a hemocytometer. Double-walled cysts which did not pick up eosin stain were taken as viable.

Synthesis of CAMP

Amebae exposed to plain nonnutrient agar (control) or nonnutrient agar containing magnesium ions and taurine (experiment) were harvested, washed free of medium by centrifugation at 800 *g*, and suspended in sterile distilled water. 5 ml of the suspension (4×10^7 amebae/ml) were mixed with 9 ml of a medium containing 6 mM glucose, 90 mM NaCl, 5 μCi adenine-8-C^{14} (specific activity, 33.8 mCi/mmole: Bhabha Atomic Research Centre, Trombay, India), 2.25 mg streptomycin sulfate, and 2250 units of penicillin G. The experimental set contained, in addition, 15 mM $MgCl_2$ and 20 mM taurine. The mixture was incubated for 2.5 hr in a metabolic shaker at 37°C (set at 80 horizontal strokes per minute). Theophylline was now added to the suspension to a final concentration of 5 mM, and the incubation was continued for a further period of 2.5 hr. The cells were then recovered by centrifugation, washed repeatedly with chilled water containing 5 mM of unlabeled adenine sulfate, and finally homogenized with 5 ml of chilled 5% w/v trichloroacetic acid. The resulting homogenate was centrifuged at 800 *g* in the cold, the sedimented material was

washed once with 5 ml of chilled 5% w/v trichloroacetic acid, and the washings were added to the previous supernatant.

The combined trichloroacetic acid extracts were mixed thoroughly with 5 vol of benzene in a separating funnel. The solvent layer after separation was discarded and the extraction with benzene was repeated four times. The aqueous layer was now subjected to extraction with 5 vol of ethyl ether in a similar manner, and the extraction step with ethyl ether was also repeated four times. The resulting aqueous layer, now free of trichloroacetic acid, was mixed with activated Norit charcoal with occasional stirring. After 5 hr of contact with the aqueous extract, the charcoal which had adsorbed by now all the nucleotides was separated by centrifugation and mixed with 10 ml of 50% v/v ethyl alcohol containing 2% v/v liquor ammonia. This procedure eluted the nucleotides into the solvent layer which was then freed from the charcoal and concentrated to 4 ml by lyophilization. 200 μg of unlabeled CAMP were added to the concentrate followed by 0.4 ml of 250 mM $ZnSO_4$ and 0.4 ml of 250 mM $Ba(OH)_2$. The suspension was centrifuged and the supernatant was subjected once more to $Ba(OH)_2$ and $ZnSO_4$ treatment, centrifuged, and the supernatant was recovered and lyophilized. The dry product was dissolved in a minimal volume of water and applied to a column (0.4 × 3.3 cm) of Dowex 50 H^+ (200–400 mesh). The nucleotide was eluted with water, and 2-ml fractions were collected. Three-fourths of the carrier CAMP added and a major part of the radioactivity incorporated were recovered in fraction numbers three to seven which were pooled and lyophilized.

The following tests were applied to establish the identity of the labeled product with CAMP: (*a*) paper and thin-layer chromatography in two or three different solvent systems; (*b*) degradation by $Ba(OH)_2$ to 3′ AMP and 5′ AMP, or $NaNO_2$ and acetic acid to cyclic inosine monophosphate, or hydrolysis by carrot leaf diesterase to adenosine. These tests were performed according to procedures described by Azhar and Krishna Murti (1971). Radioactivity counts were taken in a Packard Liquid Scintillation Spectrometer essentially as described by Hadi and Krishna Murti (1967). *Assay of phosphodiesterase* (Orthophosphoric diester phosphohydrolase EC 3.1.4.1) was assayed according to the procedure described by Butcher and Sutherland (1962) using CAMP as substrate.

Protein content of extracts was estimated colorimetrically (Lowry et al., 1951).

RESULTS

Induction of Encystation by CAMP

The action of CAMP in inducing the encystment of *H. culbertsoni* is illustrated in Fig. 1. In a

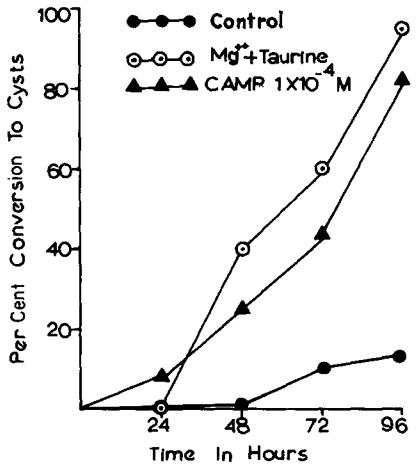

FIGURE 1 Induction of encystation of *H. culbertsoni* by CAMP. Encystation was followed axenically as described in the text. Mg^{2+} (15 mM) and taurine (20 mM) or CAMP (0.1 mM) were incorporated in the nonnutrient agar. A sample of cell population containing a minimum of 100–150 trophozoites or cysts was used in triplicate for hemocytometric counts. The results plotted are the means of two experiments.

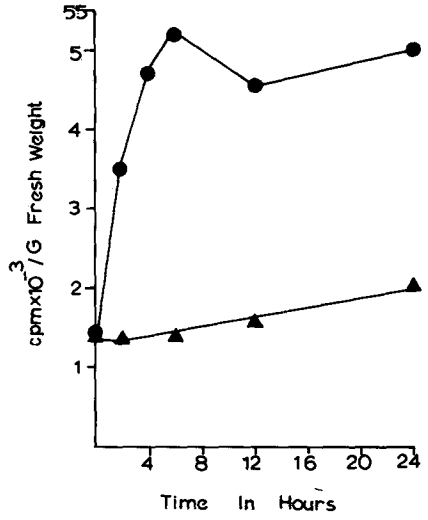

FIGURE 2 Synthesis of CAMP by *H. culbertsoni* during exposure to encystment medium. Experimental conditions as described in the text. 1 g wet weight contained about 2 × 10^8 trophozoites. The cpm plotted represents the label incorporated into CAMP isolated from 1 g of weight of cells and processed as described in the text for radioactivity counting. ●——●, experimental; ▲——▲ control.

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number of repetitions of the experiments, a similar pattern of results was obtained. CAMP was effective even at a concentration of 1×10^{-5} M and gave about 60% encystment within 72 hr. 75–90% of the cysts formed by exposure for 72 hr to 1×10^{-4} M CAMP gave a negative stain with eosin. At a concentration of 10^{-4} M, 3'AMP, 5'AMP, and adenosine diphosphate (ADP) gave 24, 14, and 32% encystment under identical conditions, whereas 5×10^{-2} M theophylline gave 90–100% encystment within 72 hr. Furthermore, encystment in the presence of theophylline occurred even in a liquid medium in which magnesium and taurine were ineffective in inducing differentiation. From the results summarized in Table I, it can be seen that both actinomycin D and cycloheximide were inhibitory to the induction of encystment by dibutyryl CAMP.

Synthesis of CAMP

Results represented graphically in Fig. 2 show the effect of magnesium ions and taurine on the synthesis of CAMP by *H. culbertsoni*. During the first 4–6 hr period of exposure to the encystment medium, the extent of incorporation of adenine-8-C^{14} into CAMP was three to four times more than in amebae which were exposed for an identical period to plain nonnutrient agar. Results summarized in Table II show that the labeled product isolated was indeed CAMP.

Release of Phosphodiesterase during Encystation

The content of phosphodiesterase in *H. culbertsoni* and the activity of the enzyme released into the medium showed significant changes with the time of exposure to the medium as evident from the results summarized in Table III. The activity of the enzyme in the amebae showed a decline in the early phase of exposure to the encystment medium. The medium in which the amebae were incubated also showed significantly more activity than the

TABLE I

Effect of Actinomycin D and Cycloheximide on Encystation of H. culbertsoni Induced by Dibutyryl CAMP

Addition	Concentration	% of encystation		
		14 hr	24 hr	40 hr
Dibutyryl CAMP	1×10^{-6} M	15	72	89
Dibutyryl CAMP	1×10^{-5} M	40	85	100
Dibutyryl CAMP	1×10^{-4} M	20	60	60
Dibutyryl CAMP	1×10^{-5} M	30	70	85
Dibutyryl CAMP + actinomycin D	1×10^{-5} M 1×10^{-7} M	6	9	50
Dibutyryl CAMP + actinomycin D	1×10^{-5} M 1×10^{-4} M	nil	9	12
Dibutyryl CAMP + actinomycin D	1×10^{-5} M 1×10^{-5} M	nil	nil	nil
Dibutyryl CAMP + cycloheximide	1×10^{-5} M 1×10^{-6} M	10	14	42
Dibutyryl CAMP + cycloheximide	1×10^{-5} M 1×10^{-5} M	nil	9	19
Dibutyryl CAMP + cycloheximide	1×10^{-5} M 1×10^{-4} M	nil	nil	nil

Results of means of two separate experiments.
Counts of trophozoites and cysts were made on triplicate samples.

TABLE II

Identification of Labeled Compound Obtained from H. culbertsoni

Solvent system	cpm applied	cpm recovered as CAMP spot
System A	1080	846
System B	1080	900
System C	1000	830

System A: butanol:acetone:acetic acid:H_2O:ammonia (14:10:6:9:1 v/v)—Ascending paper chromatography.
System B: butanol:methanol:ethyl acetate:ammonia (7:3:4:4)—ascending paper chromatography.
System C: 1 M ammonium acetate:95% v/v ethanol by thin-layer chromatography (75:30 v/v).

Treatment	cpm applied	cpm recovered	
		Compound	Activity
Sat. $Ba(OH)_2$*	1860	3'AMP	950
		5'AMP	700
Acetic acid and sodium nitrite	630	CIMP	600
Carrott leaf enzyme (*Daucus carota*)	1330	Adenosine	1000

* The products of $Ba(OH)_2$ treatment, viz. 3'AMP and 5'AMP were identified by paper chromatography using solvent system sat. ammonium sulphate:1 M sod. acetate:isopropyl alcohol (80:18:2).

medium in which controls were incubated for an dientical period.

DISCUSSION

The results of the present study lead to the inescapable inference that CAMP is the mediator by which the metabolic machinery of *H. culbertsoni* is geared for differentiation. Exogenously added CAMP took almost the same time needed by magnesium ions and taurine to bring about encystment. The failure of CAMP to shorten the period of encystment could be due to: (*a*) the limited permeability of the nucleotide or (*b*) its quick degradation by phosphodiesterase. Since dibutyryl CAMP also took the same time for inducing encystment as CAMP, lack of permeation can be ruled out as the limiting factor. Destruction by phosphodiesterase secreted into the medium can also be excluded, unless it is assumed that the CAMP synthesized *in situ* by the activation of adenyl cyclase by magnesium and taurine is secreted into the medium. Since theophylline, a known inhibitor of phosphodiesterase, also caused induction of encystment, the biochemical events that are triggered during differentiation are presumably regulated by the relative concentrations of CAMP and the phosphodiesterase acting on it.

Experiments not reported in this communication have shown that when the trophic form was exposed to the encystation medium for a period of 4–6 hr and subsequently transferred to plain nonnutrient medium devoid of magnesium and taurine, encystment occurred none the same. As demonstrated in the present study, magnesium and taurine are able to stimulate synthesis of CAMP in the ameba. Magnesium ions are also needed for the specific binding of taurine-S^{35} into the membranes of *H. culbertsoni* (Raizada and Krishna Murti, unpublished observation). Presumably, such a binding of taurine to the membrane leads to the activation of a particulate-bound adenyl cyclase.

The role of CAMP in the morphogenesis of the slime mold ameba is evident from the reports of Konijn et al. (1967), Barkley (1969), Chassy et al. (1969), Bonner (1970), and Murray et al. (1971). From the data presented by Hsei and Puck (1971) for Chinese hamster cells and by Johnson et al. (1971) for normal fibroblasts, CAMP also seems to be mediating the differentiation of mammalian cells. Dobrogosz and Hamilton (1971) have ad-

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TABLE III

Phosphodiesterase Activity of H. culbertsoni during Encystation

Medium	Time of exposure to medium (hr)						
	0	2	4	6	10	12	24
Nonnutrient medium control							
Cells	170	166	76	45	80	70	60
Medium	nil	133	64	not done	77	77	67
Nonnutrient medium containing 15 mM $MgCl_2$ and 20 mM taurine							
Cells	170	104	50	46	76	50	59
Medium	nil	90	78	not done	44	56	44

2 ml of suspension containing 1×10^8 cells per Petri dish were incubated at 26°C, and at indicated times cells were recovered by washing the plates with distilled water and centrifuging the suspension. The cells were then homogenized in a Potter-Elvehjem homogenizer with distilled water. The agar medium was also homogenized with water and centrifuged to give an aqueous supernatant containing the enzyme secreted into the medium. Assay of phosphodiesterase was conducted with homogenates of cells and medium as such. Reaction mixture in a final volume of 1 ml contained: 0.36 μmole CAMP, 1.8 μmole $MgSO_4$, 36 μmoles Tris HCl buffer pH 7.5; 0.5 ml homogenate or medium. Incubation at 37°C for 1 hr. Activity expressed as nmole 5'AMP formed/60 min per mg protein.

duced evidence for the involvement of CAMP in the chemotaxis of gram-negative bacteria, whereas Yokota and Gots (1970) find that the nucleotide is concerned with the formation of flagella in coliform bacteria. As shown by Chang (1968) and Riedel and Gerisch (1971) for the slime mold ameba and by the present study with *H. culbertsoni*, both of these protozooan species appear to secrete phosphodiesterase into the medium. In the slime mold CAMP stimulates development of the stalk, whereas in *H. culbertsoni* the nucleotide activates sporogenesis or cyst formation. In both cases, there is presumably an underlying phase of massive depletion of reserve food material with the concurrent synthesis of new species of RNA. The new RNA may code the enzymes needed for the development of the stalk in the slime mold and the enzymes needed for the elaboration of the cyst wall in *H. culbertsoni*. Induction of cellulose synthetase and hexosamine monophosphate aminotransferase does occur during the exposure of *H. culbertsoni* to the encystment medium (Raizada and Krishna Murti, 1971).

Glucose and oligosaccharide primers needed for the synthesis of cellulose and mucopolysaccharide may be expected to originate from the breakdown of glycogen or other reserve polysaccharides. Degradative changes during the encystment of *H. culbertsoni* have been quantitated and will be reported elsewhere. Against the background of the well-established role of CAMP in the regulation of carbohydrate metabolism (Sutherland and Robison, 1969), it is tempting to suggest that CAMP by undergoing changes in its intracellular concentration may regulate the rates of degradation of reserve polysaccharides in starving *H. culbertsoni* and channel the products toward the synthesis of polymers which are needed to construct the cyst wall.

SUMMARY

CAMP incorporated into a nonnutrient agar medium mimicked the combined action of magnesium ions and taurine in bringing about the differentiation of trophic *Hartmannella culbertsoni*, a free-living soil ameba, to mature cysts. The encystation induced by CAMP was inhibited by actinomycin D and cycloheximide. Amebae exposed to magnesium ions and taurine in the nonnutrient medium synthesized three to four times more CAMP than amebae exposed to plain nonnutrient medium.

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