Phytochemistry, 1975, Vol. 14, pp. 903–907. Pergamon Press. Printed in England.

A POSSIBLE ROLE OF CYCLIC 3′,5′-ADENOSINE MONOPHOSPHATE IN THE GERMINATION OF *CICER ARIETINUM* SEEDS*

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(*Revised Received* 23 *June* 1974)

Key Word Index—*Cicer arietinum*; Leguminosae; germination; cyclic AMP; cyclic AMP phosphodiesterase; protein phosphokinase; RNase; protein synthesis.

Abstract—Changes in the activities of adenyl cyclase, cyclic AMP phosphodiesterase, protein phosphokinase, RNase, protease, DNA, RNA and protein synthesis during the initial imbibition phase of the germination cycle of *Cicer arietinum* (chick pea, Bengal gram) are reported. Activation of adenyl cyclase and phosphorylation of cellular proteins appears to precede RNA and protein synthesis in the imbibed seeds.

INTRODUCTION

The sequence of the complex biochemical events leading to embryo growth has not been fully unravelled and very few leads are available on the nature of the agents that trigger the renewed development of the embryo during seed germination [1]. Recent studies on weed seed germination suggest that after imbibition hormonal factors have to be released from a bound or storage form to stimulate the synthesis of regulatory molecules [2]. A number of reports implicate cyclic 3′,5′-adenosine monophosphate as one of these regulators presumably acting as a second messenger for plant hormones [3–6]. This communication shows that there is a marked spurt in the activity of adenyl cyclase in the early phase of germination of *Cicer arietinum*. During the period studied, protein phosphokinase activity of germinating seeds increases, but activities of a phosphodiesterase acting on cyclic AMP, protease and RNase remain unchanged. The rise in activity of adenyl cyclase and protein phosphokinase is also accompanied by increased synthesis of RNA and proteins but not DNA.

* Communication No. 1861 from the Central Drug Research Institute, Lucknow, India.

RESULTS

Adenyl cyclase and cyclic AMP phosphodiesterase activity during germination

In Fig. 1 are shown the results of a study of incorporation of adenine-8-^{14}C into cyclic AMP. Under these conditions the increase in the incorporation

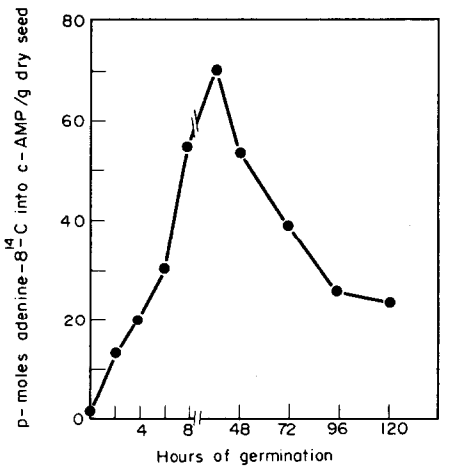

Fig. 1. Adenyl cyclase activity during germination of *Cicer arietinum* seeds. One g seed sections were shaken at 37° for 2·5 hr in a buffered medium containing NaCl 140 mM, KCl 5 mM, $MgSO_4$ 2 mM, Tris–HCl buffer (pH 7·5) 15 mM, glucose 6 mM, adenine-8-^{14}C 5 μCi, streptomycin 2·25 mg and penicillin G 2250 units. After this initial incubation, theophylline was added to the medium to a concn of 5 mM and flasks shaken for a further period of 2·5 hr. Cyclic AMP was then isolated as described earlier [4].

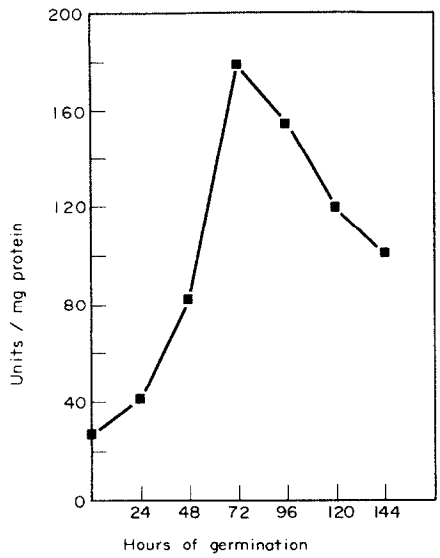

Fig. 2. Cyclic AMP phosphodiesterase activity during germination of *Cicer arietinum* seeds. The reaction mixture in a final vol of 0·9 ml contained acetate buffer (pH 5·0) 50 μmol, cyclic AMP 0·36 μmol, $MgSO_4$ 1·8 μmol, snake venom (Naja Naja) 200 μg and enzyme. After incubation at 37° for 1 hr, the reaction was stopped by addition of 0·1 ml 55% TCA, and in the protein-free supernatant Pi was estimated colorimetrically. One unit is the amount of enzyme that mediates the release of 1 nmol of Pi from cyclic AMP in 60 min.

of adenine-8-[^{14}C] into cyclic AMP-[^{14}C] can be taken as an index of adenyl cyclase activity [7, 8]. The labelling of this nucleotide is significant within 15 min of imbibition and the extent of incorporation steadily increases with time reaching a peak value around 24 hr of germination.

The intracellular concentrations of cyclic AMP in intact cells would be affected by both the rate of synthesis and the rate of its degradation. Results presented in Fig. 2 reveal that a phosphodiesterase (acting on cyclic AMP *in vitro* assay) is also present in the dormant seed; its activity does not change greatly up to 24 hr of germination but then registers a rapid increase reaching a peak value around 72 hr. This phosphodiesterase activity is not inhibited by 1 mM theophylline which is known to inhibit over 60% the activity of mammalian cyclic AMP phosphodiesterase [9].

In another set of experiments attempts were made to assay adenyl cyclase activity under *in vivo* condition. For this the seeds were imbibed in presence of adenine-8-[^{14}C] and allowed to germinate. Samples were withdrawn at intervals, cyclic AMP was purified and radioactivity in cyclic AMP was estimated. It is expected that under these *in vivo* conditions, cyclic AMP-8-[^{14}C] formed from prelabelled ATP-8-[^{14}C] would show an increase if the synthesis of cyclic AMP proceeds at a rate faster than that of its degradation. The results showed that under the conditions employed there was a steady increase in radioactivity of cyclic AMP with the time of germination from 36 pmol at 0 hr to 123 pmol at 8 hr.

Protein phosphokinase activity during germination

Since cyclic AMP cyclic AMP mediates its regulatory function by phosphorylating target proteins [10], it was of interest to determine the extent of protein phosphorylation during germination of *C. arietinum*. This was carried out by following the incorporation of ^{32}P from *in situ* generated [γ^{32}P]-ATP into purified total cellular proteins. Under the conditions increase in the ^{32}P incorporation into nucleotide free protein reflects the level of protein phosphokinase activity. This activity in *C. arietinum* seeds increases very significantly and reaches a peak value around 6 hr as shown in Fig. 3.

Synthesis of DNA, RNA and protein during germination

Seeds imbibed at 4° for 5 hr in sterile water containing thymidine-[^{3}H] or uracil-2-[^{14}C] or *Chlorella* protein hydrolysate-U-[^{14}C] were

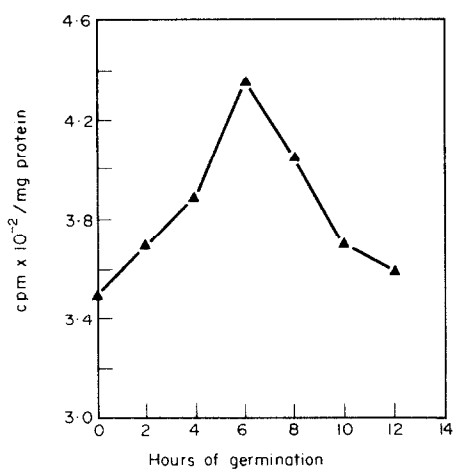

Fig. 3. Protein phosphokinase activity during germination of *Cicer arietinum* seeds. Experimental conditions were as given under Table 1.

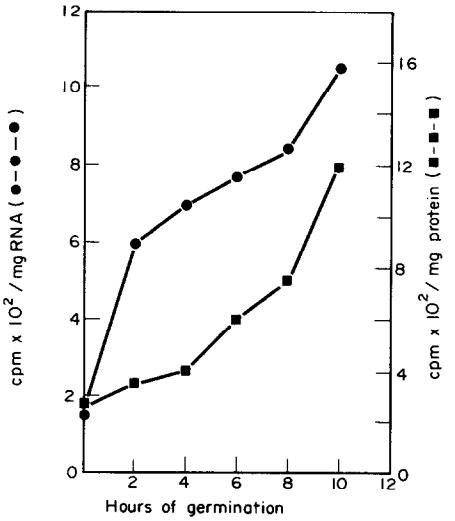

Fig. 4. Initiation of RNA and protein synthesis during germination of *Cicer arietinum* seeds. RNA ●—●; Protein ■—■. Fifty seeds were placed in 100 ml Erlenmeyer flask containing 50 ml uracil-2-^{14}C or *Chlorella* protein hydrolysate-U-^{14}C (5 × 10^6 cpm/ml) and left at 4° for 5 hr. After this seeds were removed, washed and allowed to germinate at 22°. Samples were taken out at different time intervals and processed for RNA or protein isolation.

allowed to germinate and DNA, RNA and protein were isolated from samples at different intervals. The extent of incorporation of thymidine-[^{3}H] into DNA was negligible up to 10 hr of germination. In contrast there was significant incorporation of uracil-2-[^{14}C] into RNA and *Chlorella* protein hydrolysate-U-[^{14}C] into proteins as shown in Fig. 4. The specific activity of RNA was found to increase threefold within 2 hr and fivefold within 10 hr of germination. The increase in the incorporation of *Chlorella* protein hydrolysate-U-[^{14}C] into proteins was not very significant up to 4 hr, but increased four-fold within 10 hr of germination.

RNase and protease activities during germination

Since the intracellular concentrations of RNA and proteins would reflect a balance of synthesis and degradation of these macromolecules the total RNase and protease activities of the seeds were assayed in *C. arietinum*. The results, however, showed that the specific activities of these two degrading enzymes did not register any significant change during 10 hr of germination following imbibition for 5 hr at 4°.

Effect of cyclic AMP and IAA on protein phosphokinase and synthesis of RNA and proteins during germination

In view of the fact that both cyclic AMP or IAA stimulates *in vitro* synthesis of RNA and proteins in *C. arietinum* seedlings [6], the effect of these two agents was studied on macromolecular synthesis and on protein phosphorylation during early phase of germination. The results presented in Table 1 reveal that cyclic AMP or IAA did stimulate RNA or protein synthesis under *in vitro* conditions as judged by the extent of incorporation of precursors into macromolecules at different period of germination. Also in Table 1 are given data which indicate that cyclic AMP or IAA during the same intervals stimulate *in vitro* phosphorylation of cellular proteins.

DISCUSSION

The ability of IAA to stimulate the adenyl cyclase activity of plants *in vitro* is evident from previous reports [4, 5]. Unequivocal evidence for the presence of cyclic AMP in the plant tissues has also been adduced [11, 12]. In the present study of the nature of the biosynthetic events in the post imbibition stage of germination of *C. arietinum*, it has been shown that among the activities stimulated are adenyl cyclase and protein phosphokinase and the synthesis of RNA and proteins, but not DNA. Assay of cyclic AMP phosphodiesterase, protease and RNase activities reveals that these enzyme activities remain unchanged during the first 10 hr. Admittedly intracellular concentrations of metabolite and macromolecules have to reflect a balance between their synthesis and degradation. In the present study adenyl cyclase activity, RNA and protein synthesis show significant increase whereas cyclic AMP phosphodiesterase RNase and protease activities do not change significantly. Even if one were to assume that the degrading enzymes were operationally active during germination, the increase in the cyclic AMP or the increased ability to incorporate uracil into RNA or amino acids into proteins would mean that the rate of synthesis is much faster than degradation under *in vivo* conditions.

Enzymes mediating the synthesis of RNA are present in dormant pea seeds, but are functionally inactive in the early phase of germination [13].

Table 1. Effect of cyclic AMP and IAA on RNA and protein synthesis and protein phosphokinase activity during germination of *Cicer arietinum* seeds

Additions (0·1 mM)	Hours of germination					
	0	2	4	6	8	10
cpm in RNA per mg seed						
None	26·6	32·0	32·4	35·3	43·3	44·3
Cyclic AMP	45·0	51·9	54·0	59·7	83·7	84·6
IAA	48·9	63·0	72·0	75·0	84·6	—
cpm in protein per mg seed						
None	10·4	11·2	13·7	19·1	—	20·9
Cyclic AMP	10·8	14·6	17·9	26·7	—	26·0
IAA	10·5	15·7	19·0	20·2	—	29·7
cpm/mg protein						
None	344	355	390	437	—	409
Cyclic AMP	445	532	571	520	—	438
IAA	585	637	691	709	—	502

1 g Samples (withdrawn from seeds imbibed in distilled water and germinated as indicated) taken out at specified intervals of germination were cut into thin sections and suspended in a medium containing 140 mM NaCl, 5 mM KCl, 2 mM $MgSO_4$, 15 mM Tris–HCl buffer (pH 7·5) and where required the indicated amount of cyclic AMP or IAA. The suspension was incubated at 37° for 2 hr and then 5 μCl or uracil-2-^{14}C or *Chlorella* protein hydrolysate U-^{14}C was added and incubation continued for 15 min. Then the seed sections were removed from the medium, washed, homogenized and processed for the isolation of RNA and protein.

For the phosphokinase activity the suspension medium contained, in addition, 6 mM glucose, 2·25 mg streptomycin, 2250 units of penicillin G, 1 mM NaF and 25 μCi carrier free ^{32}P and was incubated for 60 min at 37° in a metabolic shaker to generate (γ^{32}P) ATP *in situ*. After 60 min 0·1 mM EDTA, 0·4 mM theophylline and where required the indicated amount of cyclic AMP or IAA were added in a final vol. of 10 ml. The contents were further incubated for 30 min, then samples processed for isolation of total proteins.

This seems to be true of *C. arietinum* as well. It is tempting to postulate the release of either IAA or other regulatory substances following imbibition as a prerequisite for the activation of transcription and the resultant translation process. That IAA at least is released from a bound to free form is implicit in two reports [14, 15]. IAA thus released can modulate transcription directly either by affecting RNA polymerase or indirectly through the agency of cyclic AMP. Cyclic AMP may in turn by phosphorylation derepress transcription by phosphorylating the histones. The exact mechanism remains to be further elucidated, nonetheless the appearance of adenyl cyclase and the activation of transcription in the post imbibition period cannot be dismissed as unrelated to each other in the synchronization of biochemical events that follow the termination of seed dormancy.

EXPERIMENTAL

Seed material. The seeds of Bengal gram (*Cicer arietinum*: chick pea) with a germination of 92–95% were used throughout.

Radioactive precursors. Adenine-8-[^{14}C] (sp. act. 29·47 mCi/mol), uracil-2-[^{14}C] (sp. act. 13·43 mCi/mmol), *Chlorella protein hydrolysate-U-*[^{14}C] (sp. act. 13 mCi/m atom) and thymidine-[^{3}H] (sp. act. 6600 mCi/mol) were obtained from Bhabha Atomic Research Centre, Trombay, Bombay, India. Thymidine-[^{3}H] and uracil-2-[^{14}C] were dissolved in H_2O and adjusted with carrier molecules to a final concn of 0·05 M containing 5×10^6 cpm/ml in each case. In case of *Chlorella* protein hydrolysate-U-^{14}C 0·1 mg casamino acids (Difco, Detroit, Michigan, U.S.A.) were added to every ml with 5×10^6 cpm.

Ingestion and seed germination. Surface sterilization of *C. arietinum* seeds was carried out by immersing them first for 5 min in 0·1% $HgCl_2$ followed by immersion for 20 min in 50% EtOH containing 200 mg/ml thymol and 500 mg/ml glycerol. The seeds were then thoroughly washed with sterile H_2O. 50 seeds were covered with 50 ml of one of the precursor solns or H_2O and left at 4° for 5 hr. After this period the seeds were removed and washed thoroughly with H_2O. These imbibed seeds were allowed to germinate. To trigger germination, the imbibed seeds were transferred to a chamber and spread on filter paper sheets moistened with double distilled H_2O and incubated at 22° in the dark for periods varying from 0 to 12 hr.

Isolation of macromolecules. Samples ingested with labelled precursors were taken out at required periods of time and were processed for the isolation of DNA and RNA according to the method described by Ogur and Rosen [16] and for the isolation of protein as described by Trewavas [17].

Adenyl cyclase was estimated by first incubating the tissue with adenine-8-[^{14}C] and then measuring the rate of labelling of cyclic AMP as suggested by Robinson *et al.* [7]. The details of the isolation and identification procedure for cyclic AMP has been described earlier [4].

Cyclic AMP phosphodiesterase. A 10% homogenate was prepared in 150 mM KCl (pH 7·2) by grinding the tissue with the

acid washed sea sand in a pre-chilled mortar. The slurry was filtered through cheesecloth and the filtrate centrifuged at 950 *g* for 10 min. In the supernatant the enzyme activity was assayed by a modified procedure of Butcher and Sutherland [9].

Protein phosphokinase was estimated, by following the incorporation of ^{32}P from *in situ* generated [$\gamma^{32}P$] ATP into total cellular proteins isolated from the seeds, according to Sahib *et al.* [18].

Ribonuclease. The assay system in a total vol of 3 ml contained 2 mg RNA, 100 μmol acetate buffer (pH 5·0) and suitably diluted enzyme. After incubation at 37° for 60 min the reaction was terminated by the addition of 1 ml of MacFadyen's reagent [19]. The tubes containing the reaction mixture were centrifuged and absorbancy of protein-free supernatant at 260 nm was measured against controls in which the enzyme was added after the termination of the reaction.

Protease. Assay system in a final vol of 3 ml contained 200 μmols acetate buffer (pH 5·0) 0·33% casein (w/v, used as a uniform suspension) and suitably diluted enzyme. After incubation at 37° for 60 min the reaction was terminated by the addition of 3 ml 15% (w/v) TCA. The tubes containing the reaction mixtures were centrifuged and absorbancy of protein free supernatant at 280 nm was measured against controls in which the enzyme was added after the termination of the reaction.

Chemical estimations. RNA was estimated colorimetrically according to Ceriotti [20], DNA according to Schneider [21] and protein by the procedure of Lowry *et al.* [22]. Inorganic phosphate was estimated according to Fiske and Subbarow [23].

Radioactive counting. Radioactivity in the samples was counted in a liquid scintillation spectrometer.

Acknowledgements The authors thanks are due to Dr. S. K. Roy and Shri Sakthi De of the Endocrinology Division for the radioactivity counts. A.K.S. and S.A. are indebted to Council of Scientific and Industrial Research, New Delhi, for grant of Research Fellowships.

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