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ADENOSINE 3′:5′-CYCLIC MONOPHOSPHATE, ADENYLATE CYCLASE AND A CYCLIC AMP BINDING-PROTEIN IN *PHASEOLUS VULGARIS*

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Key Word Index—*Phaseolus vulgaris*; Leguminosae; bean; adenosine 3′:5′-cyclic monophosphate; cyclic AMP; adenylate cyclase; cyclic AMP binding-protein.

Abstract—The validity of using the binding-protein method for determining cyclic AMP in purified and partially purified extracts of *Phaseolus* tissues has been examined and confirmed. Measurement of cyclic AMP concentration by binding-protein gave similar results to those obtained by direct spectrophotometry of purified extracts. A cyclic AMP binding-protein and adenylate cyclase were demonstrated in *Phaseolus* extracts. Isolated intact chloroplasts were shown to possess adenylate cyclase activity but persistent cyclic AMP phosphodiesterase activity obviated quantitative assessment.

INTRODUCTION

The occurrence and physiological role of 3′:5′-cyclic nucleotides in mammals [1] and in bacteria [2] is fully documented. Much of the early evidence for the presence of cyclic AMP in higher plants was presumptive, being based on the observed physiological effects of the exogenously supplied nucleotide (e.g. ref. [3]). Demonstrations that administered phytohormones elevate cyclic AMP concentrations (e.g. ref. [4]) were mostly dependent upon equivocal analyses. More recently, occurrence of cyclic AMP in a green plant has been demonstrated by a procedure involving sequential chromatographic and electrophoretic steps shown to separate cyclic AMP from all other naturally occurring adenine nucleotides [5]. The concentration of cyclic AMP found by this procedure was estimated spectrophotometrically. Other, later, estimations have used methods based on measurement of bioluminescence [6], protein kinase activity [6], competitive binding protein [7], and radioimmunoassay [8]. However, using similar procedures, Keates [9], Amrhein [10], and Niles and Mount [11] failed to detect cyclic AMP. Lin [12] has suggested that higher plants do not require cyclic nucleotides and asserts that no reliable proof has yet been furnished for the occurrence of cyclic AMP in plants. Amrhein [13] has expressed similar views and concluded that even if cyclic AMP is present, its concentration is unlikely to exceed 10 pmol/g fr. wt.

The aim of the present work was to examine the validity of applying to plant extracts the cyclic AMP-specific binding-protein method of determining cyclic AMP concentrations [14]. Demonstration of the validity of the method in the presence of plant extracts would enable a comparison to be made with results previously obtained by extraction, purification and direct spectrophotometric assay of cyclic AMP [5]. A second objective was to examine for adenylate cyclase in *Phaseolus* extracts and in particular in isolated chloroplasts.

RESULTS AND DISCUSSION

Effect of Phaseolus *extract on binding capacity of cyclic AMP binding-protein*

Under the conditions used in the present work for the determination of cyclic AMP concentrations by the binding-protein procedure [14], the standard curve intercepts the abscissa at a value of −3 pmol (Fig. 1). This value corresponds to the total amount of cyclic AMP-[8-^{3}H] present [15]. Addition of an aqueous extract of *Phaseolus* seedlings (fraction I), obtained by first dialysing the crude extract and then heating the non-diffusible fraction to 100° (see Experimental) did not alter this intercept and a linear relationship remained. However, the slope of the curve decreased indicating an increase in binding capacity. This could be due to occlusion or non-specific binding of the cyclic AMP by plant protein, or to the presence of an endogenous specific binding-protein in the extract. As discussed below, the latter is at least partially the case. In the presence of a partially purified extract, previously incubated with cyclic AMP phosphodiesterase to remove endogenous cyclic AMP, the intercept remained the same but the binding capacity was reduced. At concentrations above 8 pmol of cyclic AMP/assay, linearity was lost. These observations indicate the presence in the plant extract of a factor interfering with the binding of cyclic AMP. After treatment with cyclic AMP phosphodiesterase to remove endogenous cyclic AMP, the final, purified fraction from *Phaseolus* seedlings (fraction VI), caused no detectable alteration to the standard plot. From this, it was inferred that the interfering substance(s) had been eliminated by the procedures leading from fraction I to fraction VI. Standard curves prepared in the presence of fractions III to VI, also previously incubated with phosphodiesterase, and with internal standards of 4 pmol of cyclic AMP/assay all gave intercepts at −7 pmol corresponding to the 3 pmol of tritiated

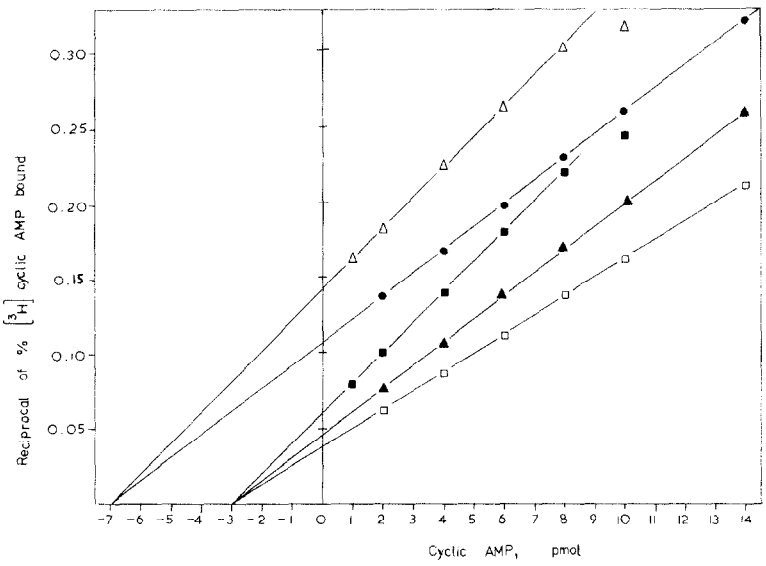

Fig. 1. Effect of *Phaseolus* extracts on binding capacity of cyclic AMP binding-protein. Standard curve ▲—▲. Binding curves determined in the presence of: fraction I □—□; fraction III ■—■; fraction VI ▲—▲; fraction III + 4 pmol cyclic AMP △—△; fraction VI + 4 pmol cyclic AMP ●—●. Each fraction had been pre-incubated with phosphodiesterase.

cyclic AMP per assay with the 4 pmol standard. Under the same conditions, the presence of fraction III caused a loss of linearity and a decrease in binding capacity, whilst addition of fraction VI caused no alteration to the standard plot.

Binding plots constructed from data determined in the presence of *Phaseolus* extracts containing endogenous cyclic AMP are shown in Fig. 2. The slope of the curve obtained in the presence of the purified fraction VI was shown to be identical to that of the slope of the standard curve. The intercepts were at −5.2 and −3.0 pmol respectively, indicating the presence of 2.2 pmol of cyclic AMP in fraction VI. The slope of the plot obtained in the presence of fraction IV was parallel to that of the standard

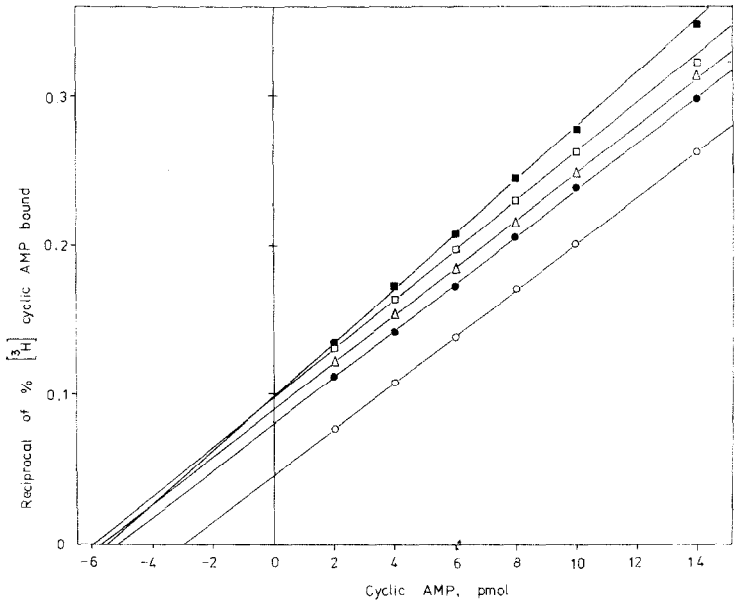

Fig. 2. Binding plots determined in the presence of *Phaseolus* extracts containing endogeneous cyclic AMP. Standard curve ○—○. Binding plots determined in the presence of fraction II ■—■; fraction III □—□; fraction IV △—△; fraction VI ●—●.

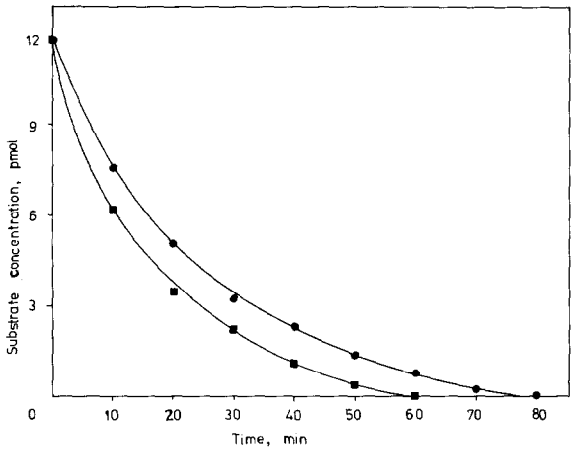

Fig. 3. Comparison of the rates of hydrolysis by mammalian cyclic AMP phosphodiesterase of an authentic sample of cyclic AMP ●—● and the extracted sample ■—■.

curve; the intercept indicates the presence of 2.5 pmol of cyclic AMP. The difference of 0.3 pmol between the cyclic AMP content of fractions IV and VI is attributable to loss in recovery during the electrophoresis procedures. Since the curves obtained with fractions IV and VI both parallel the standard curve, it was concluded that neither fraction interferes with the assay. In contrast, the presence of fraction II and to a lesser extent fraction III, caused a change in the slope of the standard curve indicating interference with the assay. These observations indicate the compatability of fraction IV onwards with the binding-protein assay procedure and mean that the cyclic AMP content of plant extracts, purified to the stage represented by fraction IV, can be reliably determined by the binding-protein method. One circumstance in which this would not be true would be if the plant extract contained a substance which mimics the binding kinetics of cyclic AMP, and so interferes with the assay without destroying the linearity of the plot. This possibility however was eliminated by the observation that incubating fraction IV with mammalian phosphodiesterase produced a closely similar hydrolysis curve to that produced by authentic cyclic AMP (Fig. 3). The validity of applying the binding-protein procedure to plant extracts purified to the fraction IV stage was further confirmed by comparing the results (2.2 nmol/g dry wt of tissue) with those obtained by direct spectrophotometry of similar extracts (2.4 nmol/g dry wt of tissue). It was thus concluded that although present work confirms the suggestion [13, 16] that plants contain substances that interfere with the binding-protein assay, it also shows that use of internal standards and purified extracts obviates this problem.

The increased binding capacity in the presence of the crude aqueous extract of *Phaseolus* (Fig. 1) is attributable to an endogenous cyclic AMP binding-protein. Examination of the binding capacity of each of a series of protein fractions obtained by fractional precipitation of a *Phaseolus* extract with $(NH_4)_2SO_4$ (Table 1) showed two active fractions, one precipitating at 0–20% saturation and the other at 40–60% saturation. Comparison

Table 1. Cyclic AMP binding capacities of protein fractions from $(NH_4)_2SO_4$ precipitation of a *Phaseolus* extract

Fraction (% saturation with $(NH_4)_2SO_4$)	Binding capacity (pmol cyclic AMP/mg protein) Charcoal method [14]	$(NH_4)_2SO_4$ method [23]
0–20	1.4	1.6
20–40	0.1	0
40–60	0.7	0.8
60–80	0	0
80–100	0	0

by two different procedures (see Experimental) indicated that these two fractions had similar binding capacities to those of preparations obtained from Jerusalem artichoke rhizomes [17] and wheat embryos [18]. Although of lower specific activity, the protein fraction precipitating at 40–60% saturation with $(NH_4)_2SO_4$ was available in greater quantity and was more stable than the 0–20% precipitate. It has consequently been used for further study. That the binding-protein in the 40–60% $(NH_4)_2SO_4$ precipitate exhibits a degree of specificity towards cyclic AMP is indicated by the results presented in Table 2. These show that while 2 μM cyclic AMP completely blocked the binding of cyclic AMP-[U-^{14}C], the other nucleotides tested had little or no effect. The only exception to this was AMP. It should be noted that the figures shown, in Table 2, for binding capacity are conservative since the 40–60% $(NH_4)_2SO_4$ precipitate is also the major cyclic AMP phosphodiesterase-containing fraction of *Phaseolus* seedlings [19]. Further studies of this binding-protein fraction are in progress.

Adenylate cyclase activity in Phaseolus *seedlings*

Having established the conditions necessary to validate application of the binding-protein assay for cyclic AMP to plant extracts, the method was used to examine for adenylate cyclase activity in *Phaseolus* seedlings. The results obtained demonstrated incorporation of radioactivity from adenine-[U-^{14}C], ATP-[U-^{14}C] and kinetin-[U-^{14}C], into cyclic AMP. The greatest incorporation (1300 cpm) was seen with adenine-[U-^{14}C]; small but reproducible incorporation (41 cpm) occurred with kinetin-[U-^{14}C] (6-furfuryladenine-[U-^{14}C]). Al-

Table 2. Cyclic AMP binding capacity of the 40–60% $(NH_4)_2SO_4$ fraction in presence of nucleotides and related compounds

Additions (final concentration 2 μM)	Binding capacity (pmol cyclic AMP/mg protein)
None	0.80
Cyclic AMP	0
Cyclic CMP	0.75
Cyclic GMP	0.80
Cyclic UMP	0.85
Adenine	0.70
Adenosine	0.80
AMP	0.30
ADP	0.60
ATP	0.70

Table 3. Incorporation of radioactivity from ^{14}C-labelled adenine, ATP, and kinetin into cyclic AMP by disrupted chloroplasts

Precursor (50000 cpm) supplied	Incubation in presence (+) or absence (−) of binding protein*	Radio-activity in supernatant (cpm)	Radioactivity incorporated into cyclic AMP cpm	%
Adenine-[U-^{14}C]	+	7520	1040	2.1
	−	6480		
ATP-[U-^{14}C]	+	8800	2810	5.6
	−	6000		
Kinetin-[U-^{14}C]	+	270	12	<0.1
	−	258		

*Commercial preparation (BDH).

Table 4. Radioactivity from cyclic AMP-[U-^{14}C] bound by the disrupted chloroplast preparation in presence or absence of exogenously supplied binding-protein*

Incubation time (min)	Presence (+) or absence (−) of bind-ing-protein	Cyclic AMP-[U-^{14}C] in super-natant [14] (cpm)	Cyclic AMP-[U-^{14}C] bound %
120 (Control I)	+	737	7.3
	−	4320	43.2
15 (Control II)	+	924	9.2
	−	6840	68.4

*Commercial sample (BDH).

though ATP is the usual substrate of adenylate cyclase, at least in animal tissues, incorporation of radioactivity from ATP-[U-^{14}C] into cyclic AMP by *Phaseolus* tissues was lower (421 cpm) than that from adenine-[U-^{14}C]. This is probably a reflection of the greater permeability of membranes to adenine than to ATP. The extensive purification procedure employed, together with the use of internal standards, make this an effective qualitative demonstration of the presence of adenylate cyclase activity in *Phaseolus* seedlings. However, the concomitant occurrence of cyclic AMP phosphodiesterase and the absence of saturating concentration of substrate, make quantitative interpretation difficult.

Demonstration of the presence of adenylate cyclase activity in isolated intact chloroplasts was effected after disrupting the chloroplasts by osmotic shock [20]. Two sets of incubation were carried out, one set in the presence of cyclic AMP binding-protein (BDH), and one set in its absence. The rationale was that the binding-protein would trap the cyclic AMP produced before the endogenous phosphodiesterase could attack it. Any unbound cyclic AMP and any other labelled nucleotide or potentially interfering compound would be removed by the charcoal adsorption step at the end of the incubation. The difference between the radioactivity of the supernatant from the two sets of incubates, i.e. those with and without adding binding-protein, is proportional to the adenylate cyclase activity. As would be expected, ATP was the best precursor of cyclic AMP (Table 3). Adenine was again incorporated but kinetin was not. These results, like those from the whole seedling, were taken as a qualitative demonstration of adenylate cyclase activity but no quantitative deductions could safely be made. To test for non-specific binding of cyclic AMP by other proteins in the *Phaseolus* extract and to demonstrate the validity of the assay, cyclic AMP-[U-^{14}C] was added to the extract in the presence and absence of exogenously supplied binding-protein. The results (Table 4) showed that after the charcoal adsorption step of the standard binding-protein procedure [14], 6–7 times more cyclic AMP-[U-^{14}C] was concentrated in the supernatant when the binding-protein was present than when it was omitted. Thus, although there is 7–9% non-specific binding of cyclic AMP by the extract, addition of the binding-protein substantially increases binding.

From the results presented, it is concluded that the specific cyclic AMP binding-protein procedure [14] for the estimation of cyclic AMP concentration can be applied to plant extracts. These extracts must, however, be partially purified, as described, and internal standards should be included in the binding assay. The close similarity of the cyclic AMP concentrations measured by the binding-protein method with those obtained by direct spectrophotometry of purified extracts affords further confirmation of the presence of cyclic AMP in *Phaseolus vulgaris*. This is endorsed by the demonstration of adenylate cyclase activity in isolated intact chloroplasts, a finding which is in agreement with other published reports [8, 21, 22].

EXPERIMENTAL

Materials. Seeds of *Phaseolus vulgaris* L. cv The Prince were surface sterilized by immersion for 1 hr in a 5% (w/v) soln of calcium hypochlorite containing 0.2% (v/v) stergene, thoroughly washed in H_2O for 24 hr and germinated in Levington compost at 25°. Seedlings were grown in a light cycle of 18 hr light (5.5 klx) and 6 hr dark. For the ensuing experiments 7- to 8-day-old seedlings were used.

Preparation of fraction I (*crude boiled extract*). Using a chilled mortar and pestle, seedlings (100 g fr. wt) were ground in ice-cold Tris–HCl buffer (50 mM; pH 7.4) containing 2 mM $MgCl_2$ and 2 mM $(NH_4)_2SO_4$. Acid-washed sand was used to facilitate grinding. The slurry was squeezed through a double thickness of gauze and the coarse debris removed by centrifuging at 200 *g* for 1 min at 0°. To remove cyclic AMP the supernatant was dialysed against Tris–HCl buffer (50 mM; pH 7.4) for 18 hr. Phosphodiesterase activity in the non-diffusible fraction was eliminated by heating at 100° for 4 min. To the heated fraction was added solid theophylline and 2-mercaptoethanol to give a final concn of 8 and 6 mM, respectively. This prepn was designated fraction I.

Extraction of cyclic AMP from seedlings. Seedlings were immersed in liquid N_2 and freeze-dried; samples (200 g) of the freeze-dried powder were homogenized in an ice-cold monophasic mixture of MeOH–$CHCl_3$–HCOOH (12:5:3). Care was taken to avoid a rise in temp. during homogenization. The homogenate was then put through the purification procedure described in ref. [5]. At each stage of the procedure, a sample was retained for examination by the binding-protein method [14]. The sample retained after the anion-exchange step on Dowex-2 was designated 'fraction II'; that obtained after the

Dowex-50 cation-exchange step was called 'fraction III'; the sample of cyclic AMP eluted after the first TLC step was 'fraction IV' and that obtained following HV-electrophoresis was 'fraction V'. The sample of cyclic AMP obtained following the final TLC step was designated 'fraction VI'. Each of the fractions was concd by evaporation *in vacuo* and then appropriately diluted to contain equal concns of the original material. The purified sample of cyclic AMP (fraction VI) was assayed spectrophotometrically [5].

Validation of the use with plant extracts of the binding protein assay for cyclic AMP [14]. Cyclic AMP was assayed by the competition for binding, between the sample and tritiated cyclic AMP, on a specific binding-protein. The procedure used was that described in ref. [14] with minor modifications; the binding-protein was obtained from BDH Ltd., Poole, Dorset. A standard binding curve was determined using 6 different concns of cyclic AMP and the separate effect of adding each of the *Phaseolus* fractions was examined. A reagent blank was included. Each point was determined in quadruplicate; reproducibility was good. Cyclic AMP standards contained 0–14 pmol/50μl and the tritiated cyclic AMP soln contained 3 pmol/50 μl. Tris–HCl buffer (50 mM; pH 7.4) was used to make up the radioactive and the non-radioactive cyclic AMP solns described and also as the buffer in which the binding assays were conducted. The sp. act. of the cyclic AMP-[8-^{3}H] was 29 mCi/μmol. Before use, the commercial sample of binding-protein was diluted so that 100 μl of the diluted soln bound 21.7% of the triated cyclic AMP in the standard assay procedure in the absence of a non-radioactive sample. As recommended in ref. [15], internal standards were employed throughout. A typical assay tube contained 100 μl of diluted binding-protein soln, 50 μl of the sample to be assayed, 50 μl of cyclic AMP-[8-^{3}H] and buffer to a final vol. of 350 μl. For each point determined, 3 controls were carried out viz; (i) adsorption efficiency control in which the tube contained 50 μl of cyclic AMP-[8-^{3}H], and 300 μl of the buffer; (ii) zero standard control containing 100 μl diluted binding protein, 50 μl of cyclic AMP-[8-^{3}H] and 200 μl of buffer; (iii) internal standard containing 50 μl of the unknown, 50 μl of a standard cyclic AMP soln, 50 μl of tritiated cyclic AMP, 100 μl of diluted binding-protein and buffer to vol. Before examining the effects of fractions I and VI on the standard assay, these fractions and their corresponding blanks were incubated with phosphodiesterase for 1 hr at 30°. The enzyme was removed by heating the fractions at 100° and then centrifuging.

Preparation of cyclic nucleotide binding-protein from Phaseolus. Seedlings (200 g fr. wt) were ground in ice-cold Tris–HCl buffer (50 mM; pH 7.4) containing 2 mM $MgCl_2$. The coarse debris was removed as above and the supernatant dialysed against the same buffer. The non-diffusible fraction was subjected to fractional precipitation with $(NH_4)_2SO_4$. Protein fractions obtained at 0–20%, satn, 20–40%, 40–60%, 60–80% and 80–100% were redissolved in the minimum vol. of 50 mM Tris–HCl buffer (pH 7.4) containing 8 mM theophylline, 6 mM 2-mercaptoethanol and 0.05 mM EDTA, then dialysed against the same buffer.

Measurement of cyclic nucleotide binding capacity. The binding capacity of each of the protein fractions was measured by precipitating the binding protein plus bound cyclic AMP-[8-^{3}H]. The procedure used was that of ref. [23] modified to employ a total vol. of 0.5 ml. As a cross-check on the results obtained, a second procedure was used in which 0.5 ml of charcoal suspension [14] was added, then centrifuged and the supernatant counted. The effect of the simultaneous presence of other nucleotides on the binding of cyclic AMP was assessed using 50 μl of 2 μM nucleotide solns.

Incubations with cyclic AMP phosphodiesterase. Samples (12 pmol) of cyclic AMP, either purchased from Boehringer, Mannheim or extracted from *Phaseolus* seedlings (fraction VI), were incubated with cyclic AMP-specific phosphodiesterase (33 akat; Boehringer, Mannheim) in a total vol. of 100 μl of Tris–HCl buffer (50 mM; pH 7.4). The reaction was terminated by addition of 55% (w/v) $HClO_4$. Each reaction was carried out in quadruplicate giving good reproducibility. The protein ppt. formed by addition of $HClO_4$ to the reaction mixtures was in each case removed by centrifuging and 50 μl samples of the supernatants assayed for residual cyclic AMP by means of the binding-protein procedure. As before, internal standards were used throughout.

Incorporation of radioactive percursors into cyclic AMP. The cut ends of excised shoots of 6-day old seedlings of *Phaseolus* were immersed in vials containing an aq. soln of the appropriate precursor or putative precursor (10^5 cpm in 3 ml). The seedlings used for these expts had been kept in the dark for 14 hr before the shoots were excised. To maximize transpiration and hence uptake of the solns, the shoots were illuminated and a slow current of air passed over them. When the soln in each vial had been taken up, 3 ml of H_2O was added. Finally, when the latter had been taken up, the excised shoots were immersed in liquid N_2, freeze dried, and the cyclic AMP contents extracted as described above. Samples were taken for examination by scintillation spectrometry.

Isolation of intact plastids. After 14 hr in the dark followed by 1 hr in the light, leaves were detached from 6-day-old *Phaseolus* seedlings. The leaf tissue (83 g) was homogenized in 300 ml of 0.3 M sucrose containing 50 mM Tris–HCl buffer (pH 7.4), 1% (w/v) cysteine, and 5 mM EDTA. Homogenization was effected using an ice-cold mortar and a plastic spatula. The homogenate was centrifuged at 200 *g* for 5 min and the supernatant layered onto a sucrose density gradient. This consisted of 25 ml of 46% (w/v) sucrose in 50 mM Tris–HCl buffer (pH 7.4) on top of 40 ml of 50% (w/v) sucrose in the same buffer. After centrifuging at 2000 *g* for 15 min, intact chloroplasts were located in a band above the interface between the sucrose layers; they were removed by pipette. Samples were examined by light microscopy and shown to consist of intact chloroplasts.

Demonstration of adenylate cyclase activity. The plastids were disrupted by osmotic shock [20] and dialysed against 50 mM Tris–HCl buffer (pH 7.4) for 6 hr at 4°. Incubations were carried out with aliquots (5 ml) of this prepn. In one set of tubes, incubation was in the presence of 1 mM adenine containing adenine-[U-^{14}C] (50000 cpm); in another set 1 mM ATP-[U-^{14}C] (50000 cpm) was used; the third set contained 1 mM kinetin-[U-^{14}C] (50000 cpm). In addition to these radioactive components, each tube contained 4 mM caffeine (0.1 ml), 4 mM theophylline (0.1 ml), 2 mM NaF (0.1 ml), 2 mM dibutyryl cyclic AMP (0.1 ml), 1% (w/v) BSA (0.1 ml), 50 mM Tris–HCl buffer (pH 7.4; 3 ml) and undiluted binding-protein soln (0.5 ml). A parallel set of incubations in which the binding protein had been omitted were also carried out. All incubations were at 31°. After 2 hr, 3 ml of charcoal suspension was added to each incubation tube and the contents agitated for 3 min before being centrifuged at 2000 *g* for 20 min. Aliquots of each supernatant were taken and their radioactivity measured. Two controls were included in this series of expts. They contained 1 mM cyclic AMP plus cyclic AMP-[U-^{14}C] (10000 cpm) in place of the precursors. One of the controls was incubated for 15 min and the other for 2 hr.

Determination of protein concentration. Protein concns were determined by the method ref. [24] using BSA to construct a calibration curve over the range 50–300 μg/ml.

Measurement of radioactivity. Aq. samples (0.1–0.5 ml) were examined for radioactivity in 5 ml of a dioxan-based scintillant, containing 2,5-diphenyloxazole, 1,4-bis(5-phenyloxazol-2-yl)-benzene and naphthalene (NE250; Nuclear Enterprises Ltd., Edinburgh). Each sample was counted ×3 for radioactivity, for 20 min.

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