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REVIEW

CYCLIC AMP AND HIGHER PLANTS

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Abstract—Despite the evidence in support, the extent of which is outlined in this review, the occurrence of cyclic AMP in tissues of higher plants has been doubted by a number of previous reviewers. Recent MS and other evidence vindicates earlier identification of an adenosine nucleotide from plant tissues as adenosine 3′:5′-cyclic monophosphate. The additional demonstration of 3′:5′-cyclic nucleotide phosphodiesterases in higher plants, together with adenylate cyclase, a specific cyclic AMP binding protein, and calmodulin, means that plants possess all the necessary components for a functional cyclic AMP-regulated system. Whether such a system does function in plants is considered as are also the reported physiological effects of exogenously supplied cyclic AMP on plant tissues.

INTRODUCTION

Adenosine 3′:5′-cyclic monophosphate (cyclic AMP) was first discovered in 1956, by Sutherland [1], as a heat-stable factor mediating the action of adrenalin and glucagon on the activation of a mammalian phosphodiesterase. With the exception of the enucleate erythrocyte, cyclic AMP has since been found to be ubiquitous in mammalian cells where it mediates the action of the short term, rapidly acting, non-maintenance hormones such as secretin, oxytocin, vasopressin, ACTH, TSH, adrenalin, noradrenalin and glucagon [2]. The binding of one of these hormones at a specific receptor site on the cell membrane activates adenylate cyclase, which synthesizes cyclic AMP from ATP. Cyclic AMP is released inside the cell where, acting as a 'secondary messenger', it binds at specific sites on the regulatory subunits of a cyclic AMP-dependent protein kinase. As a result, this protein kinase becomes capable of phosphorylating key enzymes, such as glycogen phosphorylase and lipase [3], thereby altering their individual activities and modifying the pattern of carbohydrate or lipid metabolism, respectively.

In a control system of this type, it is essential that the concentration of the signal compound can be rapidly decreased. This is achieved by the enzyme cyclic AMP phosphodiesterase which catalyses hydrolysis of cyclic AMP to AMP. The phosphodiesterase is also subject to allosteric control [4].

In 1965 Makman and Sutherland [5] found cyclic AMP in cells of *E. coli*. At the time, this was a surprising observation since cyclic AMP was known mainly as a 'secondary messenger' mediating the action in animal tissues of hormones, which are not involved in bacterial metabolism. Pastan and Perlman [6] later showed that in bacteria, cyclic AMP is bound by a specific receptor protein concerned with initiating transcription of glucose repressive operons such as the *lac* operon. Thus, cyclic AMP is not only a 'secondary messenger' in animal tissues but also functions as a messenger in regulating gene expression in bacteria.

In the slime mould *Dictyostelium discoideum*, cyclic AMP has been shown to function as an 'acrasin', i.e. an aggregation factor during the amoeboid stage of development. Aggregation begins when a small number of the total population start to release pulses of cyclic AMP [7]. The released cyclic AMP becomes bound to receptors on the plasma membrane surface of the responding amoeba. Evidence indicates that the aggregation mechanism involves an effect by cyclic AMP on the permeability of the cell membrane to Ca^{2+} which, in turn, affects contraction and relaxation of the actomyosin network, causing formation of pseudopodia.

In view of the distribution and now well-established metabolic roles of cyclic AMP, adenylate cyclase and phosphodiesterase in animals and micro-organisms, it is pertinent and timely to consider the evidence for their existence and possibly similar physiological roles in higher plants.

OCCURRENCE OF 3′:5′-CYCLIC AMP IN HIGHER PLANTS

It is now more than ten years since the first reports of the occurrence of cyclic AMP in the tissues of higher plants were published [8–11]. These early reports, however, did not readily gain acceptance and were criticized as being based either upon indirect, presumptive evidence derived from observed physiological effects of exogenously supplied cyclic AMP, or on equivocal chromatographic identification. Among the approaches adopted, Pollard [8] had supplied germinating barley seeds with [8-^{14}C]adenine and showed that the radioactive, putative cyclic AMP obtained was inseparable from carrier cyclic AMP in ten chromatographic systems. His report also described the hydrolysis of the compound to a mixture of 3′-AMP and 5′-AMP and its deamination to cyclic IMP, which was identified chromatographically. Salomon and Mascarenhas [10, 11] extended this approach using *Avena* coleoptile tissue. Narayanan *et al.* [9] used a method, based on that of

Johnson *et al.* [12], involving use of a cyclic AMP phosphodiesterase to produce AMP from their putative cyclic AMP; AMP was determined enzymically.

A serious criticism of some of the early procedures was that the chromatographic and electrophoretic systems used would not have distinguished between isomeric nucleotides such as 2′:3′-cyclic AMP and 3′:5′-cyclic AMP. This means that what was considered to be radioactive cyclic AMP could have been 2′:3′-cyclic AMP originating from the supplied [^{14}C]-labelled purine via incorporation into RNA and subsequent degradation by ribonuclease. Identification of cyclic AMP by enzymic methods was also criticized. The basis of this criticism was that such methods are only reliable when the enzyme preparation used has been shown to have absolute specificity for the substrate.

In 1973, we were able to report [13] the application of a stringent chromatographic and electrophoretic procedure to extracts from the tissues of *Phaseolus vulgaris* and the subsequent unequivocal identification of cyclic AMP from this higher plant source. The procedure, which involved using five PC solvent systems, three TLC solvent systems and three HV-electrophoresis systems, was shown to be capable of separating all known adenine nucleotides including the 2′:3′- and the 3′:5′-isomers of cyclic AMP. Despite the stringency of this method, however, some authors [14–16] have claimed that it is not unequivocal and that there may be an adenine nucleotide which 'mimics' cyclic AMP, both chromatographically and in the cyclic AMP-specific binding protein assay [17] (see next section). They have made no suggestion, however, as to the structure of their putative pseudo-cyclic AMP. The reluctance to accept that, like other living systems, higher plants contain cyclic AMP has had a substantial deterrent effect on research in this potentially important area of plant biochemistry.

Using our earlier published procedure [13], we have recently re-isolated a sample of cyclic AMP from the leaves of *Phaseolus vulgaris* and subjected it to examination by mass spectrometry. The results [18] unambiguously confirmed our previous identification of the compound as cyclic AMP. Quantitative estimation by spectrophotometry and binding protein assay of this purified compound gave close agreement [19]. Further confirmation of the identification of a plant nucleotide as cyclic AMP has subsequently been obtained by others using mass spectrometry (MS) [20, 21] and IR [20], and by ourselves [19] and by Ashton and Polya [22] comparing the kinetics of hydrolysis with those of an authentic sample.

CONCENTRATION OF CYCLIC AMP IN PLANT TISSUES

Various estimates have been made of the concentration of cyclic AMP in plant tissues. Some of these have to be discounted for lack of specificity or non-validity of the method used. However, despite the diversity of tissues, the different authors, and the various methods used, most of the remaining estimates (Table 1) show a remarkably similar order of magnitude. This finding further supported the earlier evidence for the occurrence of cyclic AMP in tissues of higher plants. It should be noted that although the concentrations found in plants are lower than those for mammalian tissues, the cyclic AMP:ATP ratio is similar.

The methods used for measuring cyclic AMP concentrations in plant tissues have been largely taken, directly or with minor modifications, from the procedures developed earlier for studies of mammalian tissues. In some work reported, the authors have taken no precautions against interference by plant secondary products, such as phenolics, and caution needs to be exercised in attempting to interpret such work.

Probably the most reliable established method, the validity of which has been confirmed by MS [18], is that of direct extraction and purification of cyclic AMP, followed by spectrophotometric determination [13]. It is, however, an extremely tedious and time-consuming process, quite unsuitable for multiple analyses. The simplest and quickest method, developed for use with mammalian tissue, is the saturation binding assay [17] which uses a specific cyclic AMP binding protein prepared from animal tissues and readily available commercially. This method involves assaying the competition between added radioactively labelled cyclic AMP and the endogenous unlabelled cyclic AMP, for binding sites on the protein. Some papers have appeared in which this technique has been applied to plant extracts but without the necessary controls against interference from secondary products. A critical experimental appraisal of the application of this method to plant extracts has recently been made [19]. The results clearly showed the presence in extracts of *Phaseolus vulgaris* of substances interfering with the assay. Nevertheless, it was demonstrated that providing a partial purification of the extract was undertaken as described, it was valid to apply the method to determination of cyclic AMP concentration in higher plants. A similar conclusion has been reached by others [22]. Recently, the use of MS to validate application of the binding protein assay to plant tissues has been extended into a GC/MS procedure for direct assay of cyclic AMP in higher plants [20, 21].

An analogous competition principle to that described above is involved in the radioimmunoassay [23] which some experimenters have used for plant tissues. In this, an antibody specific for cyclic AMP is employed. A third type of cyclic AMP assay utilizes the stimulation of a cyclic AMP-dependent protein kinase; the radioactivity transferred from [γ-^{32}P]-labelled ATP to the protein substrate is measured [24]. Another method which has been employed for assaying the cyclic AMP content of plant tissues is a bioluminescence procedure [12] in which cyclic AMP is enzymically converted to ATP and the latter determined by a luciferin–luciferase assay.

Although most estimates of the cyclic AMP content of plant tissues are of a similar order (Table 1), some authors have reported minute concentrations close to, or below, the sensitivity of their methods [14, 25, 26]. As pointed out above, however, steps have not always been taken to ensure that methods employed have been appropriately adapted for plant extracts.

It has been suggested [44] that bacteria make the major contribution to the amounts of cyclic AMP associated with the tissues of higher plants. That this is not the case has been indicated by the work of Ashton and Polya [22] who undertook a bacterial count of the plant tissues they examined. They reported that of the total cyclic AMP concentration they found (2–6 pmol/g wet wt) for this higher plant material, less than 0.002 pmol/g wet wt could be accounted for by bacteria. Subsequent work [45] with axenic cell cultures of rye grass confirmed this view, which

Table 1. Reported concentrations of cyclic AMP in various plant tissues

Ref.	Plant species (tissue)	Concentration			Assay method
		pmol/g wet wt	pmol/g dry wt	Other units	
27	*Acer*	123–140			PK and Biolum.
22	*Agave*	1			BP and PK
28	*Avena* (coleoptile)	20–170			BP
29	*Avena* (etioplast)			67 pmol/mg	BP
30	*Citrus limeltrodes*	230–245		protein	BP
30	*Cucumis sativus*	216–252			BP
31	*Cucumis sativus*	53			BP
30	*Datura*	132			BP
27	*Daucus*	125–161			BP and Biolum.
28	*Glycine* (callus culture)	2–40			BP
32	*Helianthus annuus*			131 nM	RIA
33	*Helianthus tuberosus*	25–62			RIA
34	*Hordeum*	209–217			RIA
22	*Kalanchoe*	2–6			BP and PK
27	*Lactuca*	222–280			PK and Biolum.
30	*Lactuca*	265			BP
30	*Lemna gibba*	1040			BP
36	*Lolium*	2–12			BP and PK
30	*Nicotiana tabacum*	122			BP
32	*Nicotiana tabacum*			142 nM	RIA
27	*Nicotiana tabacum*	70–102			BP and Biolum.
37	*Nicotiana tabacum*		400		RIA
38	*Nicotiana tabacum*	70			BP and RIA
13	*Phaseolus vulgaris*		2.2		Spec and BP
39	*Phaseolus vulgaris*	8–31			BP
31	*Pinus radiata*	9.6–38.4			BP
30	*Pisum sativum*	370			BP
35	*Robinia* (sap)			9.0 nM	BP
40	*Sinapis alba*	114			BP
30	*Triticum vulgare*	170–180			BP
32	*Vinca rosea*			125–126 nM	RIA
41	*Zea*	2.1–3.5			PK
42	*Zea*		0.5–4.0		BP
43	*Zea*	35			BP
20	*Zea*	382–710	4.8–8.9		GC/MS
21	*Nicotiana tabacum* (callus culture)	84–149			GC/MS

PK = protein kinase method; BP = binding protein saturation assay; Biolum. = bioluminescence method; RIA = radioimmunoassay; Spec = spectrophotometric determination.

is also supported by studies with axenic cultures of soybean callus tissue [28] and of tobacco cells [37].

THE ENZYMES OF CYCLIC AMP METABOLISM

Cyclic AMP phosphodiesterase

Even before the early reports of the occurrence of cyclic AMP in plants, published work had begun to appear in which was described the presence in plant tissues of cyclic nucleotide phosphodiesterase activity. Amongst the plants examined for this enzyme were pea seedlings [46, 47], potato [48, 49], soybean callus tissue [50], Jerusalem artichoke [51], barley seedlings [52], carrot leaves [53], tobacco pith [54], *Phaseolus* seedlings [55, 56], and algae [57].

Several of these reports were interpreted by their authors as being indicative of a regulatory role by cyclic nucleotides in plants. However, it was argued by others that the properties of the cyclic AMP phosphodiesterase activity were so markedly different from those of its animal and bacterial counterparts that it was more likely to be concerned with the hydrolysis of 2′:3′-cyclic AMP formed during RNA catabolism. In support of this latter view, the enzyme obtained from pea seedlings by Lin and Varner [46] was reported to exhibit maximal activity at a significantly lower pH than those from animals and bacteria; it had a greater activity towards 2′:3′-cyclic AMP than to the 3′:5′-isomer, and produced 3′-AMP as the main hydrolysis product. It was also found to be insensitive to methylxanthines. In addition, the phosphodiesterase activity of barley seeds [52], carrot [58] and *Nicotiana* [59] was reported to have activity with 2′:3′-cyclic AMP at least equal to that with 3′:5′-cyclic AMP as substrate. A comprehensive survey of pH optima and substrate specificity of phosphodiesterase activities in plants, ranging from algae to higher plants, led Amrhein [57] to conclude that 3′:5′-cyclic AMP is most unlikely to be the physiological substrate of higher

plant cyclic nucleotide phosphodiesterase. However, more recent findings concerning the properties of higher plant cyclic nucleotide phosphodiesterase refute this opinion. The enzyme extracted from *Phaseolus vulgaris* seedlings and studied by Brown *et al.* [56] has a pH optimum much more like that of the corresponding mammalian enzymes [60] than that described by Lin and Varner [46]. Further, the *Phaseolus* enzyme activity hydrolyses cyclic AMP to a mixture of 3′- and 5′-AMP with the latter predominating; it is competitively inhibited by methylxanthines and has a K_m within the range of values observed for the mammalian enzyme [60]. A major difference between the *Phaseolus* enzyme and that obtained from pea seedlings [46] was observed to be that the former is active towards a number of 3′:5′-cyclic nucleotides but is inactive towards 2′:3′-cyclic AMP. These observations suggest that the *Phaseolus* enzyme is specifically involved in the metabolism of 3′:5′- rather than 2′:3′-cyclic AMP.

During our work with the cyclic nucleotide phosphodiesterase activity of *Phaseolus vulgaris*, it was observed that an endogenous activator of the enzyme was also present in extracts of these seedlings [55, 56]. The activator was shown to be a protein but the effect was demonstrably not due to non-specific stabilization of the enzyme; furthermore, it activated bovine brain phosphodiesterase to an extent comparable to its effect on the *Phaseolus* enzyme [56]. Stimulation of the phosphodiesterase from *Phaseolus* seedlings by the activator was found to be potentiated by Ca^{2+} ions and also by abscisic acid [56]. Isoelectric focussing of the two phosphodiesterase fractions (1 and 2) obtained from *Phaseolus* seedlings showed each to consist of a major and a minor component. In the presence of the activator, only a single band, with the same pI as that of the original main component, could be detected in either case.

The foregoing observations on the proteinaceous activator, especially the potentiation by Ca^{2+} of the stimulatory effect, are reminiscent of the effects of calmodulin on the comparable animal enzyme. Since we first reported these observations [56], calmodulin has in fact been shown to occur in higher plants [61, 62]. The biochemistry of the Ca^{2+}-binding protein calmodulin, which activates a number of animal cyclic nucleotide phosphodiesterases and is known to play a major role in the regulation of cellular metabolism in animal tissues, has been reviewed recently by Klee *et al.* [63] and by Scharff [64]. At this time, little is known of its involvement in plant metabolism but it will be of interest to see if it turns out to be the same as the activator of plant phosphodiesterase described above.

Examination of the subcellular distribution of cyclic AMP phosphodiesterase in seedlings of *Spinacia oleracea* [65, 66] showed that the enzyme of highest specific activity is located in the peroxisomes but that an enzyme of relatively high specific activity also occurs in the chloroplast and golgi fractions. The largest total activity of any subcellular fraction is that of the microsomes. During this study, a phosphodiesterase preparation was obtained and purified from the 100 000 *g* supernatant of a spinach seedling homogenate and shown to be resolved by gel-filtration into two major peaks of activity (peaks 1 and 2). Peak 1 was further separated into 5 protein fractions, two of which (1_c and 1_m) exhibited cyclic nucleotide phosphodiesterase activity. More detailed examination of the subcellular distribution revealed that chloroplast phosphodiesterase consists of two separable activities corresponding to peaks 1_c and 2. The microsomal, peroxisomal and golgi fractions, on the other hand, consist of phosphodiesterases corresponding to peaks 1_m and 2.

Whereas the activity of peak 1_c was shown to be greater towards purine 3′:5′-cyclic nucleotides than towards the 2′:3′-isomers, the converse was found to be true of the peak 1_m activity [66]. The pH optima of 1_c and 1_m are 6.1 and 4.9 respectively. Theophylline inhibits 1_c to a significantly greater extent than it does 1_m; Ca^{2+} ions stimulate 1_c but have no effect on 1_m. It was noted that although pre-incubation of 1_m with trypsin reduced its activity, similar treatment of 1_c resulted in a 5-fold stimulation. Freezing and thawing experiments revealed the association of 1_c, but not that of 1_m, with an endogenous inhibitor. Evidence was also obtained [66] for the presence of an endogenous activator.

Since the phosphodiesterase 1_m was found in several extra-plastidic sites and as Lin and Varner [46] had in their work used a gentler method of extraction together with a preliminary step involving centrifuging at 10 000 *g* for 10 min, it is possible that they discarded most of peak 1_c activity in a sedimented chloroplast fraction. This would explain their observations on the enzyme from pea seedlings and their conclusion that the activity is involved in the catabolism of RNA rather than in the metabolism of cyclic AMP.

In a more detailed examination of the cyclic nucleotide phosphodiesterases of spinach chloroplasts and microsomes [67], the enzymes were partially purified. The 1_c activity, extracted from the envelope fraction of intact chloroplasts, fractionated into two bands with apparent MWs of 1.87×10^5 and 3.7×10^5, respectively. Both also exhibited the activities of acid phosphatase, ribonuclease, nucleotidase and ATPase. The 1_m cyclic nucleotide phosphodiesterase from the microsomal fraction was extracted and separated into two active bands. These had MWs of 2.63×10^5 and 1.28×10^5 respectively and exhibited ATPase, ribonuclease and nucleotidase activity but not that of acid phosphatase. The partially purified 1_c was shown to have high activity towards 3′:5′-cyclic AMP and 3′:5′-cyclic GMP but little towards 2′:3′-cyclic nucleotides. In contrast, the partially purified 1_m activity was highest towards 2′:3′-cyclic AMP, 2′:3′-cyclic GMP, and 2′:3′-cyclic CMP with little activity towards 3′:5′-cyclic nucleotides. *p*-Chloromercuribenzoate inhibited 1_c but not 1_m. Electrophoresis, after dissociation with urea, showed that both 1_c and 1_m are multi-enzyme complexes. After dissociation, it was possible to reassociate the former but not the latter. The results obtained from these studies of the phosphodiesterases are compatible with the 1_c activity being involved in the metabolism of 3′:5′-cyclic AMP and with the 1_m complex being concerned with RNA catabolism. In the latter context, it was shown that the ribonuclease of the 1_m complex hydrolyses RNA to yield as its main products, 2′:3′-cyclic nucleotides.

Similarly, Ashton and Polya [48] have demonstrated the occurrence of three phosphodiesterases in potato. One has major activity as a NAD pyrophosphatase, another has greatest activity with 2′:3′-cyclic AMP as substrate, but the third has greatest activity with 3′:5′-cyclic AMP as substrate. More recently, it has been shown [68] that in *Portuluca* there are three fractions of phosphodiesterase activity capable of hydrolysing 3′:5′-cyclic AMP and 3′:5′-cyclic GMP. Fractions 1 and 2 both exhibit

Michaelis–Menten kinetics; one shows more activity towards the 3′:5′-cyclic nucleotides and the other shows more activity towards the 2′:3′-cyclic nucleotides. The third fraction exhibits positive co-operativity and is sensitive to allosteric regulation by nucleotides. Presence of cyclic GMP triggers an enzymic activity with a high activity towards 3′:5′-cyclic AMP and 3′:5′-cyclic GMP; papaverine triggers high activity towards 2′:3′-cyclic AMP.

Adenylate cyclase

The occurrence of adenylate cyclase in the tissues of higher plants was first reported in 1970 [8], being indicated by incorporation of [8-^{14}C]adenine by barley aleurone layer into a fraction co-chromatographing with cyclic AMP. Several similar demonstrations with different plant tissues soon followed [10, 28, 69, 70] and led to speculation that the adenylate cyclase of higher plants may be activated by IAA and/or gibberellins. However, identification of the enzymic product as cyclic AMP has been questioned; e.g. Alvarez *et al.* [71] have pointed out that barley seed extracts are capable of synthesizing adenosine 5′-phosphofluoridate and this may give false positive results in assays where fluoride has been added to inhibit phosphodiesterase. On the basis of negative experiments, some authors have claimed that adenylate cyclase does not exist in plants [72, 73]. Only two of the earlier studies in which positive results were obtained involved attempts to show direct conversion of ATP to cyclic AMP by a cell-free system [74, 75]. Using homogenates of maize coleoptiles, Janistyn [74] demonstrated that IAA (0.1 mM) increased cyclic AMP synthesis by 110–140 %. Giannattasio and Macchia [75], working with homogenates of Jerusalem artichoke tubers, reported that adenylate cyclase activity was increased by 300 % in the presence of mM concentrations of gibberellic acid.

The major problems in adenylate cyclase assays are product identification and quantification. Phosphodiesterase is nearly always a contaminant of adenylate cyclase preparations and hydrolyses the product of the cyclase action, i.e. cyclic AMP, making accurate determinations difficult. In analogous animal studies, caffeine and theophylline are used as potent inhibitors of the contaminating phosphodiesterase but from a practical point of view the plant cyclic nucleotide phosphodiesterases are not effectively inhibited by these methylated xanthines. A second contaminant enzyme which militates against accurate quantitative assay of adenylate cyclase activity is ATPase which competes with the former for the ATP substrate.

Work in our laboratory has succeeded in demonstrating the presence of adenylate cyclase activity in *Hordeum* and *Phaseolus* seedlings and in cell-free enzymic preparations obtained from these seedlings [19, 76]. The identity of the product was confirmed in each case using a dual-labelling isotope technique in conjunction with our chromatographic and electrophoretic method (described above). With the cell-free preparations, the problem of contaminating ATPase activity was alleviated by having present an ATP-regenerating system (phosphocreatine and creatine phosphokinase). Using this approach, adenylate cyclase activity was shown to be present in chloroplasts, isolated in an intact state, where it is associated with the membrane fraction. Demonstration of the activity in isolated chloroplasts necessitated first disrupting them, presumably because of permeability problems. In our more recent work, a cyclic AMP binding protein was also incorporated into the adenylate cyclase assay system so as to sequester the cyclic AMP produced and thus to protect it from residual phosphodiesterase activity. This demonstration of the presence of adenylate cyclase in the chloroplast confirms the earlier observation of Wellburn *et al.* [29] that *Avena* etioplasts convert [^{14}C]adenine into cyclic AMP. Location of the enzyme in a membrane fraction is compatible with the cytochemical demonstration of its presence in the plasma membrane, endoplasmic reticulum and nuclear membrane of maize [77].

CYCLIC AMP BINDING PROTEIN

In the primary messenger system of micro-organisms, a central role is played by a protein which specifically binds cyclic AMP and then initiates a chain of metabolic events [6]. In the secondary messenger system of mammalian tissues, there is also a central role for a protein specifically binding cyclic AMP; in the latter case the protein is a regulatory unit in a cyclic AMP-dependent protein kinase [78]. Presence of 3′:5′-cyclic AMP binding activity in plants was first demonstrated by Anderson and Pastan [79] working with oats. In animal tissues, such binding activity is associated with the activation of protein kinases but although protein kinases have been detected in higher plants [80, 81], these enzymes have been reported not to be activated by cyclic nucleotides. Carratu *et al.* [82] extracted a protein kinase from wheat embryos and found this not to be sensitive to cyclic AMP but later work with the same tissues [83] showed that after extensive purification, the cyclic AMP binding fraction eluted from the DEAE-cellulose column at the same point as an active protein kinase. A parallel observation has also been made with tubers of Jerusalem artichoke [84]. Whilst these latter two reports do not necessarily indicate a cyclic AMP-dependent protein kinase, they do underline the need for caution before assuming that there are no cyclic AMP-dependent kinases in higher plants. This view is also supported by the discovery of a cyclic AMP-inhibited protein kinase in a slime mould [85] and the known specificity of the animal protein kinases for their substrates. Only a few substrates have been tried in seeking the plant enzymes and then they have mostly been substrates of animal origin, such as bovine serum albumin. Furthermore, it must be borne in mind that only a relatively small proportion of the protein kinases of animal tissues are cyclic nucleotide-dependent.

Another possibility, considered in the concluding section of this review, is that the binding protein found in higher plants may have properties and functions analogous to those of the cyclic AMP binding proteins of micro-organisms rather than those of the corresponding proteins in animals. In this connexion, a highly specific 5′-nucleotidase has been extracted from wheat seedlings and potato, and shown to be competitively inhibited by 3′:5′-cyclic AMP and other cyclic nucleotides [83, 86, 87]. These higher plant 5′-nucleotidases have μmolar K_i values for 3′:5′-cyclic AMP and 3′:5′-cyclic GMP. The potato enzyme is similar with respect to MW, subunit composition and K_d values for 3′:5′-cyclic AMP and 3′:5′-cyclic GMP, to the 3′:5′-cyclic AMP receptor protein of *E. coli*; the plant enzyme and the microbial protein also behave similarly during purification [86].

Our work with *Phaseolus* [19] and other tissues [88] has also shown the presence of cyclic AMP binding activity amongst proteins extracted from higher plants. More recently, the partial purification and properties of a specific cyclic AMP binding protein from seedlings of *Hordeum vulgare* have been described [89].

Purification involved fractional precipitation, gel-filtration on Sephadex G-25 and anion-exchange chromatography on DEAE-cellulose. It resulted in a 130-fold purification. Further resolution on Sephadex G-150 yielded a single peak of cyclic AMP binding activity corresponding to an apparent MW of 1.7×10^5. The pH optimum for binding is at pH 6.5, with a sharp fall in activity below pH 6 and above pH 7.5. Under optimal conditions, maximum binding occurred at 60 min and half-maximum binding was at 15 min.

The specificity of the binding-protein from *Hordeum* was such that even at a concentration of 60 × that of cyclic AMP, no binding occurred of 2′:3′-cyclic AMP, 2′-AMP, N^6,2′-*O*-dibutyryl cyclic AMP or the 3′:5′-cyclic nucleotides of xanthine, thymidine and 2′-deoxythymidine. The binding protein was observed to have some small affinity for 3′-AMP, 5′-AMP, ADP, adenosine, 3′:5′-cyclic dAMP and 3′:5′-cyclic GMP but to a significantly lesser extent than for cyclic AMP. Interestingly, with 3′:5′-cyclic IMP there was negligible binding at low concentrations but significant binding at the higher concentrations. By a Scatchard plot of equilibrium binding data, the dissociation constant for the cyclic AMP binding protein complex was calculated to be 8 mM. This is of the same order as that (2–3 nM) of a typical mammalian cyclic AMP binding protein, i.e. that of bovine skeletal muscle [90]. On the evidence of the K_d values, the binding protein extracted from barley seedlings binds cyclic AMP much more tightly than does the protein obtained from Jerusalem artichoke tubers [84] and that extracted from wheat seedlings [83].

CYCLIC AMP AND THE PHYSIOLOGICAL PROCESSES OF HIGHER PLANTS

Elucidation of the secondary messenger role of cyclic AMP in animal tissues naturally led to speculation that this nucleotide may play a similar part in mediating the effect of phytohormones. Many attempts have been made to test this possibility, mostly by examining the effects of exogenously supplied cyclic AMP, or N^6,2′-*O*-dibutyryl cyclic AMP, on plant tissues. Following one of the earliest of these attempts, Pollard [8] reported that in barley aleurone layers GA_3 stimulates incorporation of [8-^{14}C]adenine into cyclic AMP. This work was criticized by others, mainly on the grounds that the adenine derivative studied had not been shown conclusively to be cyclic AMP. However, a more recent investigation in which the cyclic AMP content of etiolated maize shoots was measured by the binding protein method and by the activation of protein kinase, confirmed that GA_3-treated tissue contains more cyclic AMP than untreated controls [41].

Working with barley seed tissue, Galsky and Lippincott [91] showed that like GA_3, cyclic AMP promotes synthesis of α-amylase. Using a similar barley system, Duffus and Duffus [92] found this promotion to be due to an effect on the GA_3-controlled release of α-amylase. Also working with barley seed α-amylase, Kessler and Kaplan [93] noted that whereas either GA_3 or cyclic AMP would induce synthesis of the enzyme, the effect of cyclic AMP but not that of GA_3 was blocked by inhibitors of DNA synthesis. Conversely, glucose inhibited the GA_3-induced synthesis of α-amylase but had no effect on the cyclic AMP-induced synthesis. Extracts from the cyclic AMP-treated endosperm exhibited a characteristic gibberellin activity which behaved chromatographically like GA_3. Appearance of this gibberellin activity was presented by inhibitors of DNA synthesis. Barton *et al.* [94] reported that induction of α-amylase synthesis in barley aleurone by cyclic AMP or by GA_3 is inhibited by abscisic acid. They noted, however, that the concentration of abscisic acid required to do this was very much less (one-fiftieth to one-hundredth) in respect of the cyclic AMP-induced synthesis than in the case of GA_3-induced synthesis and concluded that the effects of abscisic acid on GA_3-induced synthesis and cyclic AMP-induced synthesis of α-amylase are independent and indirect. The antagonism of abscisic acid towards cyclic AMP-induced and GA_3-induced synthesis of α-amylase was also seen in work with rice endosperm [95] as was the apparent much greater sensitivity of the cyclic AMP effect to inhibition by abscisic acid.

Earle and Galsky [96] described cyclic AMP as having an essentially similar stimulatory effect to GA_3 on the production of ATPase by barley seed tissues. Similar findings were made concerning the effect of GA_3 and cyclic AMP on the protease and acid-phosphatase of the same tissues [97]. Induction of the acid-phosphatase of barley endosperm by either GA_3 or cyclic AMP was found to be inhibited by abscisic acid [98]; cycloheximide and 6-methylpurine also inhibited. Induction of isocitrate lyase in hazel seeds has been shown to be promoted by cyclic AMP [99]. Similarities were noted here, too, concerning the effects of the cyclic nucleotide and of GA_3. Of a variety of nucleotides tested only cyclic AMP consistently promoted high levels of isocitrate lyase. Marriot and Northcote [100] made similar observations concerning cyclic AMP, GA_3 and isocitrate lyase from castor beans. They noted, however, that a relatively high concentration of cyclic AMP (1–5 mM) was necessary to simulate the effect of GA_3. Cyclic AMP was reported to increase nitrate reductase activity in mung bean roots [101] but this work was later criticized and the results attributed to a non-specific effect of nucleotides [102]. Another enzymic activity reported to be stimulated by cyclic AMP is the polyphenol oxidase of callus cultures of carrot [103]. This effect is, however, specific for cyclic AMP and is antagonized by actinomycin D. The author of the latter report concludes from his studies that cyclic AMP stimulates transcription of the structural gene for polyphenol oxidase.

Reported effects on RNA metabolism include an immediate enhancement of RNA synthesis in cytoplasmic pre-ribosomal and heterogeneous nuclear RNA following treatment of gibberellin-sensitive maize protoplasts with 10^{-7} M cyclic AMP. The effects of cyclic AMP and GA_3 were found to be very similar in this respect [104]. In cowpea seedlings (*Vigna sinensis*), GA_3 and cyclic AMP were observed to increase ribonuclease activity and to augment production of ribonuclease isoenzymes [105]. Both RNA synthesis and protein synthesis were said to be involved in the responses.

Two cases of enzyme inhibition by cyclic AMP have been reported. In one, the enzyme is a wheat seedling 5′(3′)-ribonucleotide phosphohydrolase and the K_i value

at pH 4–5 is of the order of 10^{-6} M [106]. In the other, a partially purified 5′-nucleotidase from potatoes was shown to be competitively inhibited by cyclic AMP and by cyclic GMP. The K_i values at pH 5 and 8 were also approximately μmolar for both inhibitors [107].

The physiological effects of GA_3 and cyclic AMP have also been linked by experiments on seed germination. Kamisaka and Masuda [108] reported that cyclic AMP and dibutyryl cyclic AMP had no effect on the dark germination of light-sensitive lettuce seeds when given alone but that cyclic AMP synergistically enhanced GA_3-induced germination. No effect of cyclic AMP was seen with kinetin-induced germination. Later, others [109] working with light-sensitive lettuce seeds, have indicated that GA_3 does promote germination of dark-grown seeds when germination is in light for 18 hr. Cyclic AMP was said to do the same but to a smaller extent. This work also confirmed the earlier reported [108] synergism between GA_3 and cyclic AMP. The synergism was observed to be reinforced by a preliminary irradiation of seeds with red light (660 nm) and it was noted that cyclic AMP increased remarkably the inhibitory effect of abscisic acid on light-induced germination [110]. Attempts to promote germination of the freshly harvested seeds of a range of common weeds by exposing them to light have been described [111] but they remained insensitive to light unless first buried for 6 months or treated with mannitol. Germination of the mannitol-treated seeds was promoted by red light, gibberellic acid and cyclic AMP. GA_3 and cyclic AMP have been reported to promote germination, in the light, of light-inhibited seeds of *Phacelia tanacetifolia* [112]. The 8-bromo derivative of cyclic AMP was even more effective than cyclic AMP in this respect. Both the GA_3-promoted and the cyclic AMP-promoted germination were inhibited by abscisic acid and by cycloheximide. Inhibition of the germination of seeds of *Cicer arietinum* by 8-aza-adenine (mM) has been reported to be reversed by cyclic AMP (10 μM) [113].

A number of studies have been described in which the growth effects of GA_3 have been related to those of cyclic AMP. Elongation growth of *Avena* coleoptile cylinders has been shown to be stimulated by GA_3 in the presence of glucose and fructose. GA_3 enhanced the stimulatory effect of IAA but reduces sucrose-dependent growth. Each of these effects can be produced by cyclic AMP and theophylline [114]. Cyclic AMP and dibutyryl cyclic AMP significantly stimulate elongation of lettuce hypocotyls. The effect of cyclic AMP in this respect was found to be less than with GA_3; other adenine nucleotides had no observable effect. Using a stress–relaxation technique to study the mechanical properties of the treated tissues, the authors of the report concluded that in stimulating hypocotyl elongation both GA_3 and cyclic AMP caused cell-wall loosening [115]. In studying soybean seedlings from which the roots had been removed, Holm [116] described the restoration of growth induced by GA_3 and its enhancement by cyclic AMP and methylated xanthines. At concentrations as low as 50 nM, cyclic AMP restored 50–60% of the growth reduction.

Other reports which link the physiological effects of cyclic AMP and of GA_3 include one describing the enhancement by those two compounds of the formation of polyribosomes in barley embryos. Cyclic AMP was said to be more rapid in its effect. No relationship was observed between the two compounds but both were inhibited in their effects by abscisic acid and this was reversed by kinetin. The authors concluded that cyclic AMP mediates the GA_3-enhancement of polyribosome formation [117].

Effects of cyclic AMP and GA_3 on pollen tube elongation have been described. One report [118] concerned the promotion of pollen (*Tradescantia paludosa*) tube emergence and elongation by cyclic AMP. It notes that GA_3 decreased and abscisic acid increased pollen tube length. A second report concerned the pollen of *Calotropis procera* [119] and decribed the promotion of the effects of cyclic AMP by IAA and kinetin; GA_3 was said to antagonize the effects of cyclic AMP. Recent work on the pollen of *Pinus densiflora* [120] has led to the finding that the cyclic AMP content of germinating pollen is higher than that of ungerminated pollen. Cycloheximide and blastocidin S inhibited germination but did not stop accumulation of cyclic AMP. The authors of this work suggest that cyclic AMP may play a part in initiating the germination of pollen.

Two papers have been published in which it is concluded that cyclic AMP does not play the role of a secondary messenger for GA_3 [121, 122]. In one [121] the authors used the binding protein assay but could not detect any variation in the cyclic AMP concentration of leaves of *Impatiens sultani* following administration of GA_3. In the other [122] the author reported that the total incorporation of [U-^{14}C]adenosine into cyclic AMP is too small to say that there is a GA_3-stimulated incorporation and he concluded that it is unlikely that synthesis of cyclic AMP occurs in barley aleurone layers.

One or two reports have appeared implicating cyclic AMP in plant pathological processes. Synthesis of terpenoid phytoalexins, induced in sweet potato roots by inoculation with pathogens, was also found to be induced by dibutyryl cyclic AMP but not by 5′-AMP. The TLC pattern of terpenoid derivatives was the same whether induced by pathogens or by the cyclic nucleotide [123]. Sela and co-workers have implicated cyclic AMP in a plant viral infection process [124]. They showed that a crude preparation of a precursor of the antiviral factor (AVF), prepared from leaves of *Nicotiana glutinosa*, could be activated by incubation in a solution containing ATP, cyclic AMP and cyclic GMP. These investigators argued that since activation *in vivo* requires presence of the TM virus, the cyclic nucleotides needed for activation must arise by a TMV-dependent mechanism, i.e. the cyclic AMP content of infected tissue would be expected to be significantly higher than that of non-infected tissue. They present data indicating that this is the case.

Light has been reported to have a marked effect on the cyclic AMP concentration within isolated etioplasts. Using the specific binding protein procedure, Wellburn *et al.* [29] found that illumination for 2 hr with white light increased the cyclic AMP concentration from 80 pmol/mg protein to 300 pmol/mg protein. In our laboratory, we have observed that dark-grown *Phaseolus* seedlings, which contain *ca* 0.6 pmol of cyclic AMP/mg dry wt, contain 25 times more (15 pmol/mg dry wt) following 18 hr illumination with white light (unpublished observation).

Replacement by cyclic AMP and dibutyryl cyclic AMP of the light requirement for betacyanin synthesis by *Amaranthus paniculatus* has been described [125]. Puromycin was found to inhibit both the effect of light and that of cyclic AMP. It was suggested that phytochrome may control the synthesis through the

agency of cyclic AMP. A later study [126] of the induction by kinetin and by dibutyryl cyclic AMP of betacyanin synthesis in *Amaranthus tricolour* showed that the effects of both inducing compounds were inhibited by actinomycin D or puromycin. The effects of kinetin and cyclic AMP were not additive but it was noted that light promotes the effect of cyclic AMP when the latter is at its optimal concentration. The investigators conclude that cyclic AMP is not a secondary messenger in light-induced betacyanin synthesis. A similar conclusion was reached by Elliot and Murray [127] who found that a wide range of purine basis and nucleotides, including cyclic nucleotides, induced betacyanin synthesis in *Amaranthus* seedlings. Induction by 6-benzyladenine or by red light was not accompanied by changes in the total cyclic AMP content of seedlings. Janistyn and Drumm [40], however, report that the concentration of cyclic AMP in the cotyledons of mustard seedlings undergoes a strong but transient decrease in response to a 5 min pulse of red light which operates exclusively through phytochrome.

Again with the secondary messenger concept in mind, a possible relationship between auxins and cyclic AMP has been considered in some laboratories. Salomon and Mascarenhas [11] reported that within 30 min, the shortest time interval studied, *Avena* coleoptile sections treated with IAA exhibited an increased synthesis of cyclic AMP. A similar communication [70] described stimulation by IAA of cyclic AMP synthesis in *Cicer arietinum*. Cyclic AMP, itself, was reported to increase markedly the elongation of excised apical, but not subapical, segments of etiolated wheat coleoptiles [128]. Exogenously supplied cyclic AMP was also found to have a significant auxin effect in delaying petiole abscission in *Coleus* [129]. Edgerton *et al.* [130] found that unlike auxin, cyclic AMP and its dibutyryl derivative had no effect on the elongation of *Avena* coleoptile sections and concluded that it is not a mediator of auxin activity. The latter authors, however, do note that their conclusion assumes that exogenously supplied cyclic AMP penetrates the tissue. More recently, Janistyn [131] described cyclic AMP as having a similar stimulatory effect to IAA on the growth of corn coleoptiles. It was observed, however, that the effect of cyclic AMP is more rapid and ceases after 3 hr. Truelsen *et al.* [132] reported that μM concentrations of cyclic AMP can substitute for auxin and cytokinin in promoting the growth of sunflower callus but that the nucleotide has no effect on the growth of callus cultures of either *Glycine max* or *Nicotiana tabacum*. Whereas cyclic AMP has no effect in promoting the growth of Jerusalem artichoke slices, it does apparently potentiate the effects of the auxin 2,4-dichlorophenoxyacetic acid [133]. Furthermore, the same report indicates that the effects of GA_3, kinetin and cyclic AMP were additive in promoting auxin-induced cell expansion. The authors of this latter work interpret their findings to indicate that cyclic AMP does not act as a secondary messenger for an auxin, gibberellin or cytokinin in promoting cell expansion.

Some association has been traced between cyclic AMP metabolism and the growth of crown-gall tumours. Yavorskaya *et al.* [134] found that cyclic AMP inhibits induction of tumour transformation in carrot tissue infected with *Agrobacterium tumefaciens*. They attribute this to inhibition, by cyclic AMP, of the transition of cells into the G_1 phase of the mitotic cyclic. Barula and Galsky [135] have noted that cyclic AMP inhibits the growth of crown-gall tumours on leaves of *Phaseolus vulgaris*. Also working with tumour tissue obtained by inoculating leaves of *Phaseolus vulgaris* with *A. tumefaciens*, Rutherford *et al.* [136] found consistently more adenylate cyclase activity in normal leaf tissue than in the tumour tissue.

Other descriptions of the effects of cyclic AMP on the physiological processes of higher plants include one on flowering and another on leaf movement. The sucrose-induced inhibition of the flowering of *Lemna* spp. has been observed to be reversed by cyclic AMP [137, 138] as has also the NH_4NO_3-induced inhibition of flowering in the same species [137]. Studies of the effect of cyclic AMP on the leaf movement of *Trifolium repens* have shown that 0.5–1 μM concentrations of the continuously supplied nucleotide lengthen the periodicity. At the higher concentration, the effect is more pronounced and the rhythm dampens out faster. The authors of these studies conclude that their results do not support the cyclic AMP model of the circadian clock proposed by Cummings [139] but that they could be due to an influence of Ca^{2+} transport [140].

Finally, in considering the physiological effects on plant tissues of cyclic AMP, the question of the permeability of plant cells to this nucleotide arises. Some experiments have been described which attempt to throw light on this problem. For example, Wiedmaier and Kull [141, 142] have shown that exogenously supplied cyclic AMP is taken up by leaves of *Impatiens* and *Petunia* and enters the cells. It is rapidly degraded both in intact tissues and in isolated protoplasts [142]. It appears that etioplast envelope membranes from greening, etiolated laminae of *Avena sativa* are relatively impermeable to GA_3, abscisic acid and cyclic AMP. However, there is a marked increase in permeability to all three compounds during the early stages of greening. This is especially noticeable after 2 hr [143].

CONCLUSION

Some previous reviewers [14–16] have concluded that there has been no unequivocal demonstration of the occurrence of cyclic AMP in higher plants. In reaching this conclusion, they have declined to accept sequential co-chromatography and electrophoresis, with an authentic sample, through 11 different systems, collectively shown to separate cyclic AMP from all other known adenosine derivatives [13]. By their criteria, few compounds have been unequivocally identified in plants. Recent mass spectrometric evidence [18] conclusively vindicates the earlier identification [13]. Other recent studies, discussed in this review, show the existence in higher plants of adenylate cyclase, cyclic AMP phosphodiesterase and a specific cyclic AMP binding protein.

The presence in higher plants of the cyclase, cyclic nucleotide phosphodiesterases and a specific binding protein means that plant tissues possess the potential for a cyclic AMP regulated system. This potential is heightened by the recent discovery in plants of calmodulin [61, 62], the Ca^{2+}-binding protein which activates a number of cyclic nucleotide phosphodiesterases in animal tissues. However, although the potential is there, whether or not the system is physiologically functional remains obscure at this time. A variety of physiological effects of cyclic nucleotides in plants have been reported, and are outlined above; there are, however, fundamental differences

between the plant hormones and those animal hormones whose activity is mediated by cyclic AMP. The plant hormones have a wider range of effects and are able to penetrate cells. Animal hormones mediated by cyclic AMP trigger the adenylate cyclase on the cell membranes and it is the nascent cyclic nucleotide, released inside the cell, which produces the specific response. A possibly more significant difference is that whereas each of the mammalian hormones which exert their influence via cyclic nucleotides is a non-maintenance hormone exhibiting characteristic short-term, rapid effects, e.g. those of adrenalin and glucagon, the longer-term, slower acting hormones responsible for growth and development act via mechanisms not involving cyclic nucleotides. Some physiological events in plants, e.g. onset of germination, seismonastic leaf movements, and the various light-related responses, may be analogous to the short-term, rapidly acting mammalian systems and could, therefore, involve cyclic AMP-mediated signalling. Although a close similarity to the mammalian system with its adenylate cyclase receptors and catalytic units strictly orientated on the cell surface would seem to be precluded for the reasons given, some phytohormonal effects may be mediated by cyclic AMP.

In considering possible functions of cyclic AMP in higher plants, comparison with micro-organisms may be more relevant. In bacteria, the 'primary messenger' role of cyclic AMP is related to the nutritional requirements of an individual cell and a similar system in an autotrophic, multicellular organism such as a higher plant would seem feasible. The existence of an intercellular cyclic AMP communication system in plants, possibly reflecting nutritional status, has already been suggested [35, 42]. The concept of the evolutionary origin of chloroplasts and mitochondria as endosymbiotic prokaryotes also suggests the possibility of cyclic nucleotides operating as signal compounds in an intracellular communication system. A system of this type operates during the amoeboid cell stage of slime moulds. Amongst the roles of such a system in higher plants could be regulation of the permeability of the organelle membrane, or even that of the plasmalemma to essential metabolites.

Analogy with the known functions of cyclic nucleotides in micro-organisms also suggests that, in higher plant tissues, these compounds may be involved in regulating gene expression. In bacteria, this is essentially effected by cyclic AMP controlling repression or synthesis of inducible enzymes according to the metabolic requirements of the cell. That cyclic AMP is involved in regulating nucleic acid synthesis in higher plants has been indicated by the work, described above, of Tarantowicz-Marek and Kleczkowski [104], and of Kapoor and Sachar [105]. Another cyclic nucleotide has been implicated in this area of plant metabolism by Lanzani *et al.* [144] who have reported that cyclic GMP stimulates polypeptide synthesis in a cell-free system from wheat embryos.

It seems unlikely that the question of the role of cyclic AMP in higher plants will be answered by experiments in which the compound is applied to plant tissues and responses sought. In many such experiments, even if the applied cyclic AMP entered the cells, it would be rapidly removed by the action of the highly active and widely distributed phosphodiesterases. In plant work, use of methylated xanthines affords little practical protection against these enzymes. Attempts to overcome the initial problems of plant cell permeability to cyclic AMP have been made by some experimenters using the less polar derivative N^6,2′-*O*-dibutyryl cyclic AMP. However, although the latter is known to penetrate animal cells and evoke the characteristic responses of cyclic AMP, it cannot be assumed that the same approach is valid for plant cells. This is an argument for the design, synthesis and testing of a series of cyclic AMP analogues which penetrate plant cell membranes and resist plant phosphodiesterases.

The answers to the questions posed in the present review must surely be sought through a clearer understanding of the regulation, properties and subcellular location of the adenylate cyclase and the cyclic nucleotide phosphodiesterases, and of the distribution, properties and metabolic effects of the specific cyclic AMP binding protein. As interest develops in calcium metabolism and calmodulin in plant tissues [145], we can also expect to see further illumination of the involvement of cyclic nucleotides in the metabolism of higher plants.

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