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Evidence for Modification of Protein Phosphorylation by Cytokinins

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Kinetin stimulated phosphorylation of protein in floated Chinese-cabbage leaf discs, but inhibited protein phosphorylation in nuclei+chloroplast extracts from Chinese-cabbage or tobacco leaves. Kinetin also inhibited protein phosphorylation in isolated tobacco nuclei or nuclei from carrot secondary-phloem tissue. Purified Chinese-cabbage leaf ribosomes exhibited protein kinase activity which was inhibited by kinetin and zeatin. The ribosome-associated kinase responded to kinetin and zeatin differently from that associated with nuclei+chloroplast preparations. Protein phosphorylation *in vitro* was not affected by adenosine 3′:5′-cyclic monophosphate, indol-3-ylacetic acid or gibberellic acid. It was only inhibited by N^9-unsubstituted purines, among which the known cytokinins were the most effective inhibitors. The results are discussed in relation to possible similarities between the effects of cytokinins in plant tissues and the effects of adenosine 3′:5′-cyclic monophosphate in animal tissues. Both compounds appear to modify the activity of protein kinases and both affect many different cellular processes.

There are many similarities between the effects of cytokinins on plant tissues (Letham, 1967*a*; Srivastava, 1967; Key, 1969; Skoog & Armstrong, 1970) and the effects of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) on bacterial and animal cells (Jöst & Rickenberg, 1971). Cyclic AMP modulates the activity of a variety of specific protein kinases in animal cells (see, e.g., Walton *et al.*, 1971; Lipmann, 1971; Greengard *et al.*, 1971). In turn the protein kinases regulate the activity of various intracellular processes by phosphorylating key proteins (Jöst & Rickenberg, 1971). To date there has been no compelling evidence that cyclic AMP controls such processes in plants (but see Duffus & Duffus, 1969; Galsky & Lippincott, 1969; Pollard, 1970; Kamisaka & Masuda, 1970; Jöst & Rickenberg, 1971; Salomon & Mascarenhas, 1971; Kamisaka & Masuda, 1971; Wood *et al.*, 1972). Because of the possibility that cytokinins might modify either the amount or the action of cyclic AMP in plants we examined the effect of cyclic AMP on Chinese-cabbage leaf discs. We could detect no effect of cyclic AMP on the expansion of floated Chinese-cabbage leaf discs. Although 6-*N*-2′-*O*-dibutyryladenosine 3′:5′-cyclic monophosphate did stimulate expansion of leaf discs, this probably resulted from degradation *in vivo* releasing N^6-butyryladenine, since the latter compound was as active as kinetin in the leaf-disc-expansion assay (Berridge & Ralph, 1969; Berridge *et al.*, 1970). A similar conclusion was reached by Dekhuizen & Overeem (1972) with a soya-bean growth assay. We have since shown that whereas kinetin stimulates the growth of *Spirodela oligorrhiza* in darkness (Letham, 1967*b*), growth of *Spirodela* is not stimulated by cyclic AMP, 6-*N*-2′-*O*-dibutyryladenosine 3′:5′-cyclic monophosphate or N^6-butyryladenine.

Because we could not show any convincing effect of cyclic AMP on leaf-disc expansion or growth of *Spirodela*, we next examined the effect of cyclic AMP and various synthetic cytokinins on phosphorylation of plant proteins both *in vivo* and *in vitro*. We report below that cytokinins affect the phosphorylation of protein in Chinese-cabbage leaf discs. Cytokinins inhibited protein phosphorylation in nuclei+chloroplast preparations from Chinese-cabbage or tobacco leaf and in nuclei preparations from tobacco leaf or carrot phloem tissue. Protein kinase(s) associated with Chinese-cabbage leaf ribosomes actively phosphorylated the ribosomes *in vitro*. Cytokinins inhibited ribosome phosphorylation *in vitro*. Cyclic AMP was inactive in these systems.

We suggest (*a*) that cytokinins control the phosphorylation, and hence the activity, of key proteins in plants; in this respect they play a similar role to cyclic AMP in animal cells, and (*b*) that cytokinins modify phosphorylation of plant ribosomal protein(s), thereby controlling ribosome activity and/or integrity.

Materials and Methods

Radioactive materials

Carrier-free [^{32}P]P_i was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. [γ-^{32}P]ATP was prepared by the method of Glynn & Chappell (1964). Freshly prepared material had a specific radioactivity of about 1 mCi/μmol. Radioactivity was measured in a Packard liquid-scintillation

spectrometer (model 3375) with the toluene-based scintillator Calfluor II (Calbiochem, San Diego, Calif., U.S.A.).

Chemicals

Kinetin, zeatin, 6-benzylaminopurine, 6-benzylamino-9-β-D-ribofuranosylpurine, adenine, guanine, cyclic AMP, 6-*N*-2′-*O*-dibutyryladenosine 3′:5′-cyclic monophosphate, indolylacetic acid and gibberellic acid were products of Calbiochem. 6-Benzylmercaptopurine, 6-benzoylaminopurine, 6-(3-methylbut-2-enyl)aminopurine, N^6-butyryladenine, 3-benzyladenine, 9-benzyladenine, 6-(4-hydroxy-3-methylbut-*trans*-enyl)amino-9-β-D-ribofuranosylpurine and 6-(3-methylbut-2-enyl)amino-9-β-D-ribofuranosylpurine were kindly provided by Dr. D. S. Letham (Australian National University, Canberra, Australia). 6-Benzylamino-9-β-D-ribofuranosylpurine 3′:5′-cyclic monophosphate was synthesized by treating cyclic AMP with benzyl bromide in dimethyl sulphoxide (G. Tener, unpublished work).

Methods

DNA and chlorophyll determination. DNA in preparations of nuclei+chloroplasts or nuclei alone was measured by the Burton-modified Dische procedure (Burton, 1956) after repeated extraction of pigments with acetone and alcohol. Chlorophyll in these same preparations was measured from the E_{652} of chlorophyll extracted into aqueous 80% (v/v) acetone.

Floated leaf discs. Chinese-cabbage (*Brassica pekinensis* Rupr. cv. Wong Bok) leaf discs were excised with a cork-borer (9mm diam.) from plants grown in pots in a glasshouse and were floated in Petri dishes under continuous lighting (600lm/ft²; 6640lm/m²) at 20°C on Vickery's solution (20ml) (Berridge *et al.*, 1970). After 24h, [^{32}P]P_i (0.5mCi) mixed with sufficient sodium phosphate buffer (pH7) to give a final concentration of 0.1mM-sodium phosphate was added in 1ml to each Petri dish. Kinetin, to give a final concentration of 46μM in water (1ml), or water alone (1ml), was then added to each dish as required. Sets of discs were removed at intervals to determine protein phosphorylation. With the above procedure kinetin consistently stimulated phosphorylation of protein, whereas stimulation was not always observed when discs were floated with kinetin immediately after excision (see the Discussion section).

Chinese-cabbage leaf nuclei+chloroplast preparations. Cell-free suspensions containing nuclei and chloroplasts were prepared from young healthy Chinese-cabbage leaves chopped with scalpel blades essentially as previously described (Ralph *et al.*, 1971). The nuclei+chloroplast preparation obtained from 10g of leaves was washed by resuspending it in 1ml of 0.4M-sucrose–50mM-tris–HCl buffer (pH7.8)–10mM-2-mercaptoethanol–1mM-$MgCl_2$ and layering it over 10ml of the same medium containing 0.5M-sucrose. The material was centrifuged at 1500*g* for 5min at 0°C and the washed nuclei+chloroplast pellet recovered. This step was repeated and the pellet finally resuspended in an appropriate volume (usually 1–2ml) of the above medium containing 0.25M-sucrose. A typical preparation contained 0.44mg of chlorophyll and 93μg of DNA.

Tobacco leaf nuclei+chloroplast preparations. Initially, cell-free suspensions of nuclei and chloroplasts were prepared from young tobacco plants (*Nicotiana tabacum* cv. White Burley) by chopping the chilled (0°C) leaf (10g) with scalpel blades and by using the method previously described (Ralph *et al.*, 1971). Subsequently, the medium and procedure developed by Kuehl (1965) for isolation of tobacco nuclei and chloroplasts was employed, with the exception that the leaf was hand-chopped with scalpel blades. The final nuclei+chloroplast pellets were resuspended in 1–2ml of 0.1M-tris–HCl buffer (pH7.8)–20mM-2-mercaptoethanol–5mM-$MgCl_2$. Kuehl's (1965) procedure gave greater yields of intact nuclei. Nuclei+chloroplast preparations made by either method were actively phosphorylated *in vitro* by [γ-^{32}P]ATP. A typical preparation from 10g of leaf prepared by Kuehl's (1965) method contained 960μg of DNA and 1.42mg of chlorophyll.

Tobacco leaf nuclei preparation. Nuclei were prepared from chilled young tobacco leaf (20g) hand-chopped in Kuehl's (1965) medium (40ml). The filtered nuclei+chloroplast suspension was adjusted to 1% Triton X-100 and centrifuged at 250*g* for 10min. The pellet was resuspended in 3ml of Kuehl's (1965) medium and diluted with 1ml of 0.1M-tris–HCl buffer (pH7.6)–20mM-mercaptoethanol–5mM-$MgCl_2$. The suspension was adjusted with Triton X-100 to 2% (v/v), layered over 10ml of Kuehl's (1965) medium and centrifuged at 250*g* for 10min. The resulting pellet was again resuspended in diluted Kuehl's (1965) medium containing 2% Triton X-100 and the procedure was repeated. The final nuclei pellet was resuspended in 0.1M-tris–HCl buffer (pH7.6)–20mM-cysteine–5mM-$MgCl_2$ (1–2ml) as required. A typical preparation contained 900μg of DNA. All steps in the isolation procedure were monitored by examining the preparations stained with 1% Aniline Blue in the appropriate medium, under oil immersion with a Leitz microscope. The final pellets also contained starch granules.

Carrot phloem nuclei preparation. The outer flesh was removed from a large fresh carrot (*Daucus carota* L.) with a vegetable peeler (Dalson double-action). Layers of secondary phloem tissue (20g) were then peeled off with the vegetable peeler

and homogenized in 0.25M-sucrose–10mM-mercaptoethanol–50mM-tris–HCl buffer (pH8)–10mM-$MgCl_2$ (40ml) at 0°C and low speed in a VirTis homogenizer for 2min. Preliminary experiments were required to determine an appropriate speed for the homogenizer. The homogenate was filtered through two layers of muslin and two layers of Miracloth, then centrifuged at 1500*g* for 10min. The orange-coloured pellet was resuspended in 2ml of the 0.25M-sucrose medium and centrifuged through a 10ml underlayer of 0.3M-sucrose in the same medium. The pellet was resuspended and the process repeated. The final pellet was resuspended in 1–2ml of 0.25M-sucrose medium as required. Visual examination of the final nuclei preparations stained with 1% Aniline Blue solution in medium showed numerous rather small nuclei, small starch grains and occasional very small fragments of orange-coloured tissue. A typical nuclei preparation from 20g of tissue contained 40μg of DNA.

Ribosome preparations. Chinese-cabbage leaf ribosomes were prepared from 50g of leaf by several cycles of differential centrifugation at 225000*g*. The procedure for obtaining these preparations and their quality have been described previously (Berridge *et al.*, 1970). For the present experiment the ribosome solutions were underlayered with 2ml of 1M-sucrose in 0.01M-tris–HCl buffer (pH7.6)–0.016M-$MgCl_2$–0.06M-KCl–0.006M-2-mercaptoethanol before centrifugation. This improved the removal of fraction I protein. The final ribosome pellets were resuspended in 2ml of buffer and the E_{260} and E_{280} determined.

Protein phosphorylation in vivo. Leaf discs. Phosphorylated protein in floated leaf discs was determined as follows. Randomized leaf discs were floated on Vickery's solution [1.7mM-$MgSO_4$–2.1mM-$(NH_4)_2SO_4$–1.05M-$CaCl_2$] in Petri dishes on medium containing [^{32}P]P_i with or without kinetin. Sets of discs were removed at intervals, extensively washed with 0.1% sodium dodecyl sulphate–0.1M-NaH_2PO_4, then with water, blotted dry, weighed and homogenized with 5ml of 10% (w/v) trichloroacetic acid containing 0.1M-NaH_2PO_4 in a Kontes Duall glass homogenizer. The total radioactivity in the discs was calculated from that in a portion of the homogenate. Other 0.5ml samples were heated at 100°C for 10min, chilled, then filtered through Whatman GF/C glass-fibre filters. The filters were well washed with 5% trichloroacetic acid containing 0.1M-NaH_2PO_4 and then dried. The hot-acid-precipitable radioactivity associated with the filters was determined. Radioactivity in serine phosphate and threonine phosphate was demonstrated by Dowex-50 column chromatography of 2M-HCl hydrolysates of the hot-acid-precipitable residue after further preparation by the procedure of Kleinsmith *et al.* (1966). A sample of authentic serine phosphate was also hydrolysed to determine a correction factor to compensate for degradation of serine phosphate during the hydrolysis. However, this correction factor probably underestimates the degradation of serine phosphate in protein. After correction, approx. 65% of the radioactivity applied to the Dowex-50 columns was eluted together with added authentic serine phosphate. A sharp peak of radioactivity was eluted from the column just before [^{32}P]P_i. The material in this peak was not identified. The hot-acid-precipitable radioactivity recovered from leaf discs was alkali-labile (100°C, 15min, 1M-NaOH) as reported for serine phosphate (Kleinsmith *et al.*, 1966).

Protein phosphorylation in vitro. Unless indicated otherwise each assay mixture contained the following materials: 10mM-$MgCl_2$; 6mM-2-mercaptoethanol; 40mM-KCl; 0.325ml of a buffer solution containing 0.25M-sucrose, 50mM-tris–HCl buffer (pH7.8), 10mM-2-mercaptoethanol and 1mM-$MgCl_2$; 0.1ml of nuclei+chloroplast or nuclei suspension or of isolated ribosomes in the above buffer solution; [γ-^{32}P]ATP (approx 1×10^7c.p.m.); and a solution of kinetin (5μg) or an equivalent volume of water to give a final volume of 0.5ml. Assay mixtures were usually incubated at 23°C for 20min.

The hot-acid-precipitable radioactivity was determined by adding 2.0ml of 10% trichloroacetic acid containing 0.1M-NaH_2PO_4 and 0.1ml of 0.1M-EDTA (pH8) to each assay tube after 20min at 23°C and heating the mixtures at 100°C for 10min. The precipitates were collected on Whatman GF/C glass-fibre filters. The filters were extensively washed with 5% trichloroacetic acid containing 0.1M-NaH_2PO_4 and 10mM-sodium pyrophosphate, dried, and the radioactivity associated with the filters was determined.

Density-gradient fractionation of radioactive tobacco nuclei + chloroplast preparations. A tobacco nuclei+chloroplast suspension was prepared from young leaf (10g) in Kuehl's (1965) medium. The final nuclei + chloroplast pellet was resuspended in 1.2ml of 0.1M-tris–HCl buffer (pH8)–20mM-mercaptoethanol–5mM-$MgCl_2$. Two 0.5ml portions were incubated with [γ-^{32}P]ATP in 2.5ml assay mixtures. One assay mixture also contained kinetin (10μg/ml). After 20min the suspensions (1ml) were layered on to discontinuous sucrose density gradients with steps of 2M-sucrose (2ml), 1.5M-sucrose (3ml), 1M-sucrose (3ml), each in 10mM-mercaptoethanol–50mM-tris–HCl buffer (pH8)–1mM-$MgCl_2$. The gradients were centrifuged at 500*g* for 10min. Two green bands of chloroplasts and some dispersed green material were observed in the gradient. The gradients were removed with a syringe from the top as four fractions (4.5ml, 3.0ml, 1.7ml and 0.8ml) and the ^{32}P radioactivity associated with protein, the total DNA (Burton, 1956) and total chlorophyll E_{652} in 80% (v/v) acetone in each fraction was determined. The middle fractions (3.0ml and 1.7ml) each contained one of the green

bands. The bottom fraction (0.8ml) was mixed before removal so that it also contained any pelleted material. In a separate experiment it was observed by light microscopy that most of the nuclei were associated with the lower green band and the pellet, although some smaller nuclei and chromatin were still present in the upper fractions.

Density-gradient fractionation of radioactive ribosomes. After incubation for 20min at 23°C, 0.3ml of an assay mixture containing 3.3 E_{260} units of ribosomes and 4×10^6 c.p.m. of [γ-^{32}P]ATP was layered on to a 5–20% (w/v) linear sucrose density gradient and centrifuged for 1.5h at 35000rev./min in a Spinco SW 50 rotor at 2°C. The sucrose solutions used to prepare the gradient also contained 0.01M-tris–HCl buffer (pH7.6)–0.01M-$MgCl_2$ and 0.01M-KCl. Then 20 fractions were collected from the bottom of the gradient and the E_{260}, the hot-acid-precipitable ^{32}P radioactivity and the hot-alkali-labile radioactivity in each fraction were determined after adding 0.1mg of bovine serum albumin to each fraction as carrier.

Determination of free phosphate in leaf discs. Two sets of randomized Chinese-cabbage leaf discs (200; 1cm diam.) were floated in Petri dishes on Vickery's solution (20ml) containing 0.5mCi of carrier-free [^{32}P]P_i and illuminated (see above). To one solution kinetin (46μM) was added. Sets of 40 discs were removed at intervals from each dish, washed extensively in ice-cold 0.1% sodium dodecyl sulphate containing 10mM-NaH_2PO_4, and then with 5×100 ml of ice-cold water. The discs were blotted dry and weighed, then homogenized with water-saturated phenol (2ml) and water (3ml). After centrifuging the homogenates at 0°C for 10min at 15000g the clear aqueous supernatants were removed and samples (0.1ml) used to measure the free phosphate present (Marsh, 1959). Other 0.1ml portions were subjected to electrophoresis together with marker [^{32}P]P_i at pH3.5 on Whatman 3MM chromatography paper to separate the [^{32}P]P_i in the extract from other radioactive products (Bieleski, 1965). After exposure of the radioactive chromatograms to X-ray film to identify the [^{32}P]P_i bands the radioactive phosphate was eluted, measured, and the total radioactivity present in the original extract as P_i then calculated. The specific radioactivity (c.p.m./μg of P_i) of the free phosphate in the discs was calculated from the results obtained.

Results

Protein phosphorylation in leaf discs

Incorporation of radioactive [^{32}P]phosphate into protein in leaf discs floated with kinetin exceeded that in discs floated without cytokinin (Fig. 1*a*). When kinetin was added to discs 5h after addition of [^{32}P]phosphate, increased phosphorylation of protein compared with untreated controls was detectable after 2h (Fig. 1*c*). Discs floated in darkness incorporated less radioactive phosphorus and did not show increased protein phosphorylation in the presence of kinetin in comparison with untreated controls. The amount of free P_i extracted from discs floated with kinetin in the light was the same as that in controls, at least for the first 12h. Subsequently the amount of phosphate extracted from the discs decreased and less free phosphate was present in discs treated with kinetin. However, the specific radioactivity of the extracted phosphate remained the same in kinetin-treated and control discs (Table 1). The results suggested that the observed effect of kinetin on protein phosphorylation did not result from an effect of kinetin on the total extractable P_i. The possibility of effects on separate minor phosphate pools was not excluded by these results.

Pratt & Matthews (1971) showed that the uptake of radioactive precursors into leaf discs is anomalous because of a wound response at the cut edges of discs. We therefore excised leaf discs from plants labelled through the roots with carrier-free [^{32}P]P_i (1mCi per plant) for 26h (Matthews, 1960) and floated these discs with and without kinetin for 20h. There was an insignificant (3%) increase in protein phosphorylation detectable in the presence of kinetin at the end of 20h. The ^{32}P radioactivity associated with protein in the original discs was extremely high, and it may have obscured a small general effect of kinetin, or a local effect at the cut edges of the discs.

Protein phosphorylation in cell-free extracts

In view of the above effects of kinetin on phosphorylation of protein in leaf discs we tested the ability of cyclic AMP and cytokinins to modify protein phosphorylation in cell-free extracts of leaf. Preliminary experiments showed that cyclic AMP was relatively stable when incubated with Chinese-cabbage leaf homogenates. Thus, when cyclic [^{32}P]-AMP was incubated with leaf homogenate for 5h at 22°C and the resulting mixture was subjected to paper chromatography, 90% of the radioactivity reamined in cyclic AMP whereas 10% was converted into AMP. In contrast [^{32}P]ATP was 90% degraded to AMP after 10min incubation with the same leaf homogenate. Nuclei+chloroplast preparations from Chinese-cabbage leaf were next incubated with [γ-^{32}P]ATP in the presence and in the absence of cyclic AMP or kinetin and the protein-associated [^{32}P]-phosphate was determined. Cyclic AMP had a very slight inhibitory effect on phosphorylation, whereas kinetin (46μM) inhibited phosphorylation of protein by 20–40% (Fig. 2). There was some variation in the exact degree of inhibition in individual experiments. The inhibitory effect of cyclic AMP and kinetin (both 46μM) was the sum of that with either compound alone. After hydrolysis with 2M-HCl of the material which survived hydrolysis with hot trichloroacetic acid, followed by column chromatography of the digest to separate serine phosphate (Kleinsmith *et al.*, 1966), 77% of the ^{32}P radioactivity

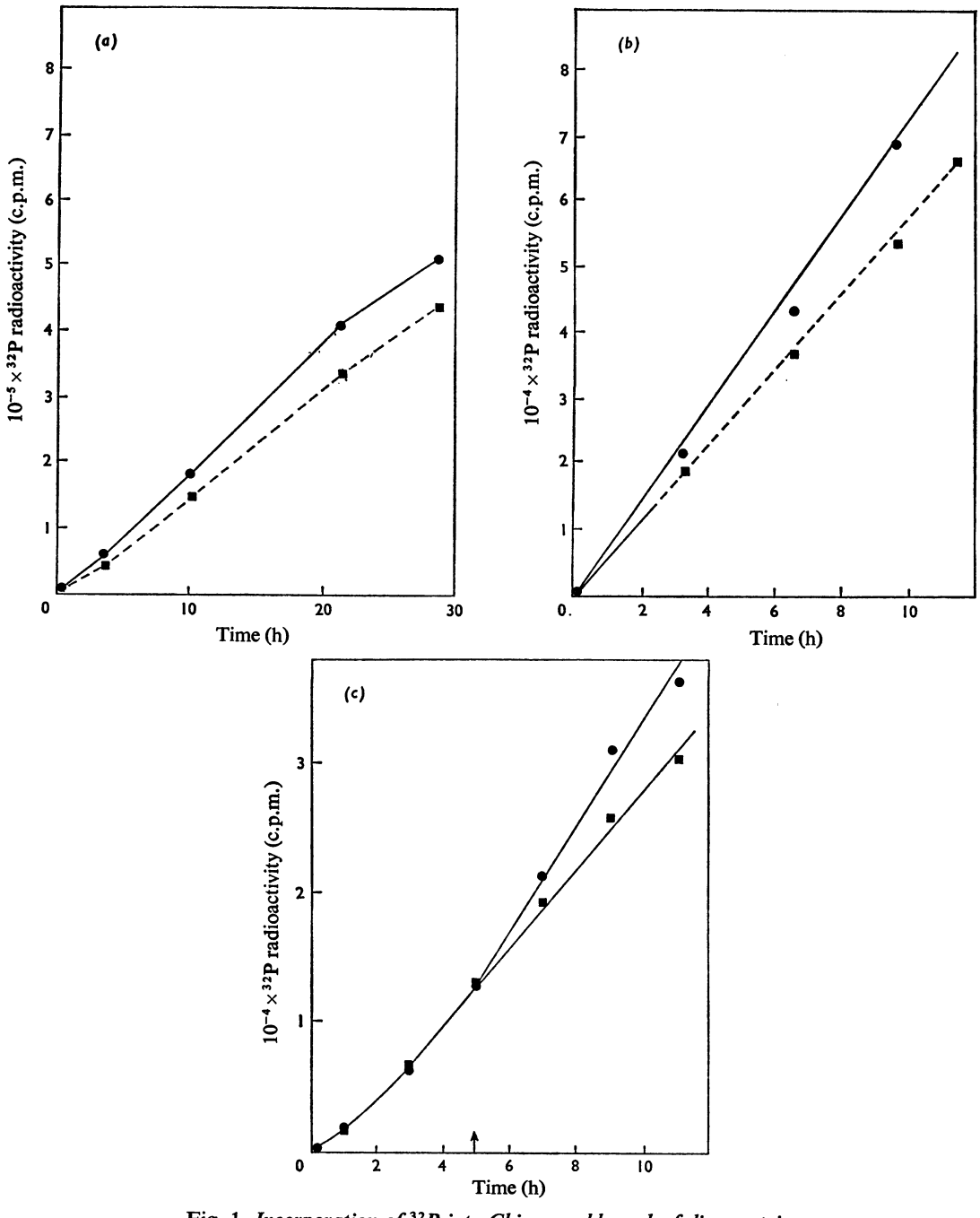

Fig. 1. *Incorporation of ^{32}P into Chinese-cabbage leaf-disc protein*

A number (60) of Chinese-cabbage leaf discs (12mm diam.) were floated under lights in each of two Petri dishes on Vickery's solution (20ml) containing 0.1mM-sodium phosphate and 0.5mCi of carrier-free $[^{32}P]P_i$ with or without kinetin. Sets of 10 discs were taken at intervals and washed, and the hot-acid-precipitable $[^{32}P]P_i$ was determined as outlined in the Materials and Methods section. (*a*) Incorporation of radioactivity into floated Chinese-cabbage leaf-disc protein immediately after excision in winter; (*b*) incorporation of radioactivity into Chinese-cabbage leaf-disc protein after floating discs for 24h in summer; (*c*) incorporation of radioactivity into floated Chinese-cabbage leaf-disc protein in winter: in this experiment kinetin (arrow) was added 5h after $[^{32}P]P_i$. ●, +Kinetin; ■, −kinetin.

Table 1. *Specific radioactivity of phosphate in extracts from Chinese-cabbage leaf discs floated on [^{32}P]P_i with or without kinetin*

For details see the Materials and Methods section.

Time floated (h)	Phosphate (μg/0.1 ml of extract)		[^{32}P]P_i (c.p.m./0.1 ml of extract)		Specific radioactivity of phosphate (c.p.m./μg of P_i)	
	+Kinetin	−Kinetin	+Kinetin	−Kinetin	+Kinetin	−Kinetin
0	1.77	1.75	—	—	—	—
4	1.76	1.77	1750	1626	986	925
8	1.76	1.75	5050	5320	2870	3040
12	1.72	1.73	8250	8097	4790	4680
24	1.18	1.31	17685	19935	15130	15220
48	1.10	1.31	22720	26880	20680	20500

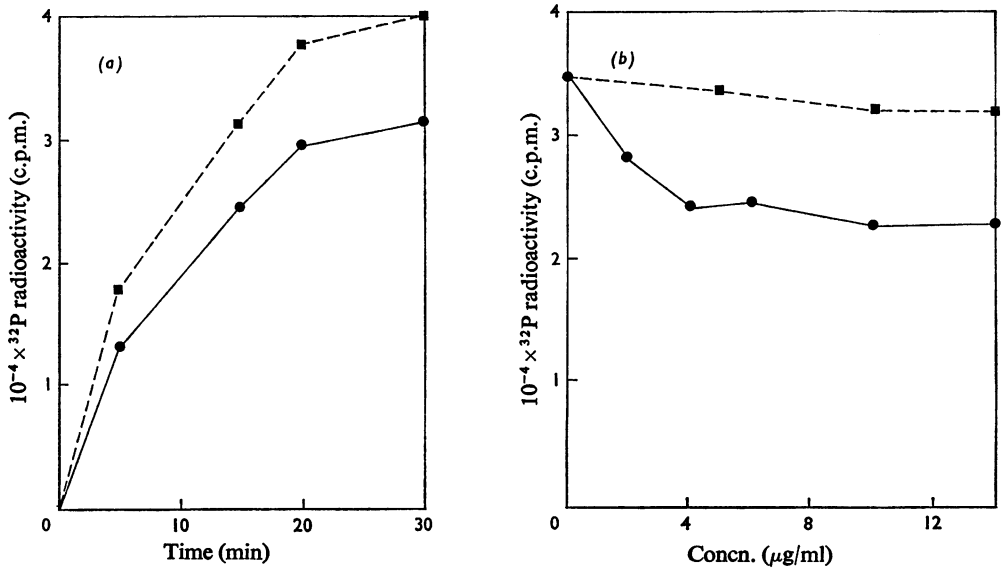

Fig. 2. *Incorporation of ^{32}P into protein in nuclei+chloroplast preparations from Chinese-cabbage leaf*

(*a*) Incorporation of ^{32}P into protein in nuclei+chloroplast preparations with and without kinetin (10μg/ml). ●, +Kinetin; ■, −kinetin. (*b*) Effect of increasing concentrations of cyclic AMP and kinetin on incorporation of ^{32}P into protein in nuclei+chloroplast preparations. ■, +Cyclic AMP; ●, +kinetin. For details see the Materials and Methods section.

was present in serine phosphate. Kinetin decreased incorporation into serine phosphate by 20–40%. Protein phosphorylation was not detected with [^{32}P]P_i as the radioactive precursor.

Phosphorylation was less if the incubation with [γ-^{32}P]ATP was carried out in darkness. However, the inhibitory effect of kinetin on phosphorylation was still evident. Nuclei+chloroplast preparations from plants placed in darkness for 72h actively phosphorylated protein in the light or dark, and the effect of kinetin was still evident. Essentially similar results were obtained by using nuclei+chloroplast or nuclei preparations from tobacco leaves, or nuclei preparations from carrot phloem tissue (Table 2). There was no difference between the effect of kinetin on protein phosphorylation in nuclei+chloroplast extracts prepared from freshly harvested leaf or from leaf discs floated for 24h. The possibility that kinetin

Table 2. *Inhibition by kinetin of protein phosphorylation in subcellular preparations from tobacco leaf and carrot tissue*

For details see the Materials and Methods section.

Expt no.	Source of material	Fraction	Kinetin concn. (μg/ml)	Radioactivity in protein (c.p.m./assay)		Inhibition by kinetin (%)
				−Kinetin	+Kinetin	
1	Tobacco leaf	Nuclei+chloroplast	10	11425	9320	19
2	Tobacco leaf	Nuclei	10	164350	103626	36
3	(*a*) Tobacco leaf	Nuclei	10	37150	30722	17
	(*b*)* Tobacco leaf	Nuclei	10	*41303	*34742	16
4	†Carrot	Nuclei	1	2020	1844	9
	Carrot	Nuclei	10	2020	1235	39

* In this assay both kinetin-treated and control samples contained actinomycin D (20 μg/ml).
† Nuclei were prepared from 10g of carrot phloem tissue for this experiment.

Table 3. *Effect of various compounds on phosphorylation of protein in nuclei+chloroplast extracts from Chinese-cabbage leaf*

For details see the Materials and Methods section. All compounds were added at 46 μM. The control assays contained 23503 c.p.m. of protein-bound ^{32}P radioactivity.

Compound	Inhibition of phosphorylation (%)
Kinetin	20
Zeatin	22
6-(3-Methylbut-2-enyl)-aminopurine	18
6-Benzylaminopurine	27
Adenine	9
Guanine	9
6-Benzylmercaptopurine	10
6-Benzoylaminopurine	12

Table 4. *Effect of cytokinin concentration on phosphorylation in nuclei+chloroplast preparations from Chinese-cabbage leaf*

For details see the Materials and Methods section.

Compound	Concentration (μg/ml)	^{32}P radioactivity in protein (c.p.m./assay)
—	—	15297
Kinetin	0.1	14129
Kinetin	1	12842
Kinetin	10	9389
Zeatin	1	12939
Zeatin	10	9025

stimulated RNA synthesis in nuclei+chloroplast extracts, thereby utilizing [γ-^{32}P]ATP and decreasing protein phosphorylation, was also examined by using actinomycin D (cf. Ralph & Wojcik, 1966). Although addition of actinomycin D stimulated protein phosphorylation slightly it did not alter the effect observed with kinetin (Table 2).

Zeatin, 6-(3-methylbut-2-enyl)aminopurine, 6-benzylaminopurine and kinetin (each 46 μM) all inhibited protein phosphorylation *in vitro* (Table 3). Kinetin and zeatin seemed to be about equally effective (Table 4). Adenine, guanine, 6-benzylmercaptopurine and 6-benzoylaminopurine (each 46 μM) were slightly inhibitory, but considerably less active than the cytokinins. 6-Benzoylaminopurine is active in the Chinese-cabbage leaf-disc-expansion assay (M. V. Berridge, R. K. Ralph & D. S. Letham, unpublished work). However, it seems possible that this compound is readily reduced to 6-benzylaminopurine in leaves. In support of this suggestion, Chinese-cabbage leaf discs floated with 6-benzoylaminopurine or 6-benzylaminopurine (each 25 μM) for 3 days expanded but did not produce roots from the ends of veins over the next 12 days, whereas discs floated with adenine or guanine (each 25 μM) expanded by the same amount as the untreated controls and produced roots. Discs floated with 6-benzylmercaptopurine expanded 34% less over 3 days than did those on 6-benzylaminopurine, yet they produced roots over the next 12 days. Adenine or guanine (46 μM) produced a 9% inhibition of protein phosphorylation whether the assays were carried out in the absence of kinetin or in the presence of 10 or 60 μg of kinetin/ml. Since the latter kinetin concentration was a large

Table 5. *Distribution of nuclei, chloroplasts and phosphorylated protein in discontinuous sucrose density gradients*

For details see the text. DNA was determined by the Burton–Dische procedure (Burton, 1956) and chlorophyll was determined from the E_{652} in 80% acetone. Fraction 1 was at the top of the gradient.

Fraction no.	Volume (ml)	DNA (μg)		Chlorophyll (μg)		10^{-3} × Protein-bound [^{32}P]P_i radioactivity (c.p.m.)	
		−Kinetin	+Kinetin	−Kinetin	+Kinetin	−Kinetin	+Kinetin
1	4.6	1.77	2.65	37	45	74.5	59.2
2	3.0	3.75	3.45	52	44	50.2	34.4
3	1.7	40.6	41.6	33	32	119.5	68.1
4	0.8	11.2	12.8	10	8	30.2	20.2

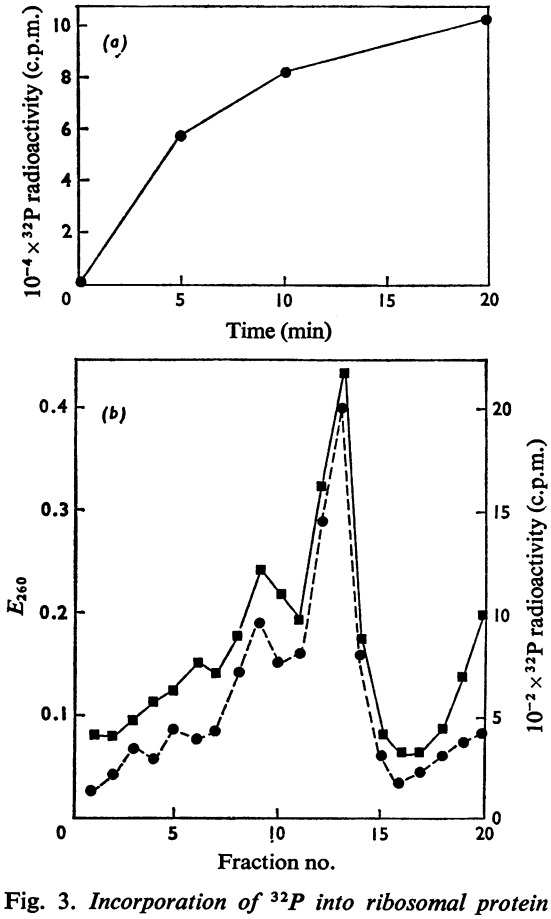

Fig. 3. *Incorporation of ^{32}P into ribosomal protein*

(*a*) Incorporation into ribosomes; (*b*) sedimentation of the radioactive ribosomes on a 5–20% linear sucrose density gradient at 4°C for 1.5h. ■, E_{260} of each fraction after dilution with 1ml of water; ●, hot-acid-precipitable ^{32}P radioactivity per fraction. For details see the Materials and Methods section.

excess (cf. Fig. 2) we concluded that the inhibitory effect of adenine or guanine on phosphorylation was additional to that of kinetin, and probably occurred at different sites.

The following compounds (46μM) were inactive *in vitro*: 6-benzylamino-9-β-D-ribofuranosylpurine 3′:5′-cyclic monophosphate, 6-benzylamino-9-β-D-ribofuranosylpurine, 6-(4-hydroxy-3-methylbut-*trans*-enyl)amino-9-β-D-ribofuranosylpurine, 6-(3-methylbut-2-enyl)amino-9-β-D-ribofuranosylpurine, 3-benzyladenine and 9-benzyladenine. Neither indolylacetic acid (1 and 10μg/ml) nor gibberellic acid (1 and 10μg/ml) affected protein phosphorylation *in vitro*.

Distribution of [^{32}P]phosphorylated protein in nuclei+chloroplast preparations from tobacco leaf

To determine whether protein phosphorylation in nuclei+chloroplast preparations was associated with nuclei or chloroplasts, tobacco leaf nuclei+chloroplast preparations were incubated with [γ-^{32}P]ATP and then fractionated on discontinuous sucrose density gradients. Some protein-associated radioactivity was present in each fraction, with most in the fraction containing the majority of the DNA. The peak distribution of radioactive protein tended to follow the peak of DNA rather than that of chlorophyll (Table 5). However, there was no clear-cut relationship between the distribution of phosphorylated protein and DNA or chlorophyll. Possibly the phosphorylated protein was released from the organelles during the incubation or centrifugation. In further experiments we found that nuclei from tobacco leaf were actively phosphorylated *in vitro* (Table 2). The tobacco nuclei were prepared by using Triton X-100 and the preparations contained no chloroplasts. Tobacco leaf was chosen for these experiments because higher yields of well-preserved nuclei were obtained from this tissue by Kuehl's (1965) isolation procedure and Triton X-100 did not

Table 6. *Effect of cytokinins and cyclic AMP on phosphorylation of Chinese-cabbage leaf ribosomes*

For details see the text. Each assay mixture contained 1.85 E_{260} units of ribosomes and 3×10^6 c.p.m. of [γ-^{32}P]ATP.

Compound	Concentration (μg/ml)	^{32}P radioactivity in protein (c.p.m./assay)
—	—	3047
Kinetin	10	2575
Kinetin	20	2276
Zeatin	10	1473
Zeatin	20	903
Cyclic AMP	40	3005

disrupt the resulting nuclei. Poor yields of nuclei were obtained from Chinese-cabbage leaf by using Triton X-100.

Phosphorylation of ribosomes

Berridge *et al.* (1970) reported that cytokinins bind to Chinese-cabbage leaf ribosomes. In view of the above effects of cytokinins on protein phosphorylation we investigated whether isolated Chinese-cabbage leaf ribosomes carried a protein kinase that was affected by cyclic AMP or cytokinins. Freshly prepared Chinese-cabbage leaf ribosomes incubated with [γ-^{32}P]ATP were rapidly phosphorylated *in vitro* (Fig. 3*a*). The radioactive material was hot-trichloroacetic acid-stable and hot-alkali-labile, as expected of serine phosphate residues in protein (Kleinsmith *et al.*, 1966). Radioactivity was largely associated with 80S and 120S material (Fig. 3*b*) when phosphorylated ribosomes were sedimented in sucrose density gradients (cf. Berridge *et al.*, 1970). Ribosome phosphorylation was not inhibited by indolylacetic acid or cyclic AMP and was only slightly inhibited by kinetin (10μg/ml). It was much more sensitive to inhibition by zeatin (Table 6). Adding small samples of the original supernatant from the ribosome preparations slightly decreased phosphorylation, as did lowering the Mg^{2+} concentration.

Discussion

In our first experiments (Figs. 1*a*, 1*c*) freshly excised Chinese-cabbage leaf discs were floated in Petri dishes on Vickery's solution (20ml) containing 0.1mM-sodium phosphate buffer (pH7) and 0.5mCi of [^{32}P]P_i with or without kinetin (46μM). Sets of discs were then removed at intervals to determine ^{32}P radioactivity associated with proteins. When the discs were taken from plants grown in the glasshouse in winter, the kinetin-treated discs immediately showed greater protein phosphorylation than controls (cf. Fig. 1*a*). However, repetition of these experiments in midsummer gave variable results. Further study showed that although Chinese-cabbage leaf discs continued to expand for a time when floated on Vickery's solution, the effect of kinetin on expansion depended on the season, age and physiological status of the plants from which the discs were taken, although kinetin invariably increased expansion of the floated discs over 3 days (cf. Berridge & Ralph, 1969). In general, the effect of kinetin was detectable earlier if discs were floated for 24h before the addition of the cytokinin. Presumably pre-floating for 24h depleted the residual endogenous supply of cytokinin in the discs so that the effect of added kinetin relative to the control was more apparent at earlier times. With these observations in view we found that more consistent results were obtained with leaf discs if they were pre-floated for 24h before addition of radioisotope and kinetin (cf. Fig. 1*b*). Others have reported that cytokinin effects vary according to season or the physiological status of the plants used. For example Mothes (1964), Richmond *et al.* (1971) and Marchetti & Baron (1971) all commented on the effects of age, seasons and other factors on kinetin activity.

We believe that our results with kinetin and leaf discs are not due to bacterial contamination, since (*a*) the discs were thoroughly washed by immersion in detergent solution and in water before homogenization, (*b*) uptake and incorporation of [^{32}P]P_i followed essentially linear kinetics, and (*c*) kinetin did not stimulate protein phosphorylation in discs floated with kinetin in darkness. Protein phosphorylation detected *in vitro* was also not due to bacterial contamination of the leaf organelle preparations, because phosphorylation was not detected when [^{32}P]P_i was used as precursor instead of [γ-^{32}P]ATP, whereas the carrot nuclei were prepared from sterile tissue and they too incorporated phosphate into protein only when incubated with [γ-^{32}P]ATP.

As yet we have no unequivocal explanation for the difference between the effects of kinetin observed in leaf discs and in cell-free leaf extracts. In view of the fact that there was no significant effect of kinetin on protein phosphorylation in discs taken from plants treated previously with [^{32}P]P_i we are inclined to discount the leaf-disc experiments as probably reflecting the well-known anomalous phosphate metabolism of excised plant tissues (cf. Pratt & Matthews, 1971). Taken together our results suggest that cytokinins may modify the phosphorylation of proteins in plants. At least two sites seem susceptible in leaves, and there appears to be some variation in the effect of different cytokinins on protein phosphorylation at these sites. The results obtained *in vitro* with various cytokinins, cytokinin derivatives, cytokinin analogues and other compounds are generally consistent with the view that only N^9-unsubstituted purines act to modify protein kinase activity. This is in accord with the

results of Hecht *et al.* (1971), who showed that N^9-ribosylation is not a prerequisite for cytokinin action. Adenine and guanine both inhibited protein phosphorylation slightly *in vitro*. Their lack of activity in the *Spirodela* growth assay and the leaf-disc-expansion assay (R. K. Ralph, unpublished work) could be due to the rapid conversion of these compounds into utilizable products (e.g. adenosine, adenosine 5′-phosphate etc.) *in vivo*. However, the full effect of adenine and guanine *in vitro* was observed even in the presence of excess of kinetin, suggesting that these compounds act at sites other than those affected by the cytokinins. In addition, the known cytokinins inhibited phosphorylation more effectively than did the other purines (Table 3), suggesting either that they bind more readily to the protein kinase(s) or that they inhibit phosphorylation at additional sites. Increasing the kinetin concentration above 5μg/ml did not inhibit protein phosphorylation proportionately in nuclei+chloroplast extracts (Fig. 2), suggesting that the cytokinins affect specific kinases rather than cause a general inhibition of phosphorylation. Further, the kinase activity associated with ribosomes showed a different sensitivity to kinetin and zeatin from that associated with nuclei+chloroplast preparations, indicating different specificities. The results obtained to date are insufficient to identify the exact site(s) of phosphorylation *in vitro* in our nuclei+chloroplast or nuclei preparations. The possibility that phosphorylation of specific histones or other chromatin-associated proteins is modified by cytokinins is attractive, since the cytokinins stimulate RNA synthesis *in vivo* (Key, 1969).

The demonstration that cytokinins modify the phosphorylation of isolated ribosomes may be related to the stabilizing effect of cytokinins on ribosomes during leaf senescence (Berridge & Ralph, 1969). It might also explain the binding of cytokinins to plant ribosomes reported by Berridge *et al.* (1970). Reversible binding of cytokinins to a ribosomal protein kinase could control the integrity and/or activity of ribosomes *in vivo* through its effect on phosphorylation. We attributed our earlier failure to detect any effect of cytokinins on protein synthesis in cell-free plant protein-synthesizing systems to the fact that cytokinins may only affect initiation of protein synthesis, since in our systems we probably detected only completion of already initiated proteins (Berridge *et al.*, 1970, 1972). If the above speculation is true it seems possible that phosphorylation of ribosomal protein(s) may play some role in the dissociation of ribosomes and/or initiation of protein synthesis. Phosphorylation of ribosomes and ribosomal proteins from animal sources has been reported, but as yet no role *in vivo* has been ascribed to the phenomenon (Blat & Loeb, 1971; Walton *et al.*, 1971). Monier *et al.* (1972) have shown that phosphorylated animal ribosomes are less active for protein synthesis *in vitro*. If cytokinins inhibit ribosome phosphorylation in plants they might thereby stimulate protein synthesis (cf. Letham, 1967*a*; Skoog & Armstrong, 1970).

Tuli *et al.* (1964) described the inhibition of hexokinase and pyruvate kinase by 6-benzyladenine. The significance of their results has been obscure and its possible relevance has only recently become apparent with the discovery that cyclic AMP acts by modifying the activity of various kinases (Jöst & Rickenberg, 1971). The multitudinous effects of cytokinins on plants (Letham, 1967*a*; Srivastava, 1967; Key, 1969; Skoog & Armstrong, 1970) are sufficiently reminiscent of the widespread and complex effects of cyclic AMP on animal tissues (see review by Jöst & Rickenberg, 1971) to suggest that the cytokinins may also modify the activity of protein kinases in their natural environment.

Our experiments do not exclude the possibility that cyclic AMP may play a regulatory role in plants as it does in animal cells. However, in the systems we have examined cyclic AMP showed no stimulatory effect on protein phosphorylation comparable with that observed in animal cell extracts. Kuo & Greengard (1969) also failed to detect cyclic-AMP-dependent protein kinase activity in plants. Plants may have evolved the cytokinins to control phosphorylation during the day–night cycle when ATP production fluctuates greatly. This situation has no direct parallel in animal cells, where the ATP supply is probably relatively constant and hormones trigger cell membrane-associated adenylate cyclase to produce cyclic AMP, which in turn modulates the action of various protein kinases. In support of this view, our preliminary experiments with nuclei+chloroplast preparations have shown that high concentrations of ATP can overcome the inhibitory effect of kinetin *in vitro*, but the inhibition can be reimposed by increasing the concentration of kinetin. Thus, cytokinin production may balance the effect of excessive ATP produced *in vivo* in daylight. It is noteworthy that kinetin has little, if any, effect on expansion of Chinese-cabbage leaf discs in darkness (M. V. Berridge & R. K. Ralph, unpublished work) when the ATP supply is presumably low. This is not true of *Spirodela* in darkness. Growth of *Spirodela* in darkness requires kinetin at least over the first 3–4 weeks after transfer to darkness (Hillman, 1957). However, the *Spirodela* culture medium contains 1% glucose, so that the ATP supply is probably substantial, even in darkness. We have found that *Spirodela* placed on water in the light for several days, then transferred to medium in darkness, continue to grow in the absence of kinetin and produce etiolated fronds. Our results would be consistent with the view that high degrees of phosphorylation tend to inhibit the growth of plants, and that cytokinins decrease phosphorylation, thereby stimulating growth. This

may be a general phenomenon, since Sheppard (1971, 1972) and Otten *et al.* (1971) have found that decreased cyclic AMP concentrations stimulate growth in animal tissues. In general, low cyclic AMP concentration would be expected to decrease kinase activity and hence phosphorylation.

G.T. was on leave from the Department of Biochemistry, University of British Columbia, Vancouver, Canada.

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