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The Binding of Kinetin to Plant Ribosomes

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The synthetic cytokinins kinetin and 6-benzylaminopurine exhibit equilibrium-type binding to purified chinese-cabbage leaf ribosomes. At 23 μM and 4°C one molecule of kinetin and 1.34 molecules of 6-benzylaminopurine are bound per ribosome. Adenine and adenine derivatives that are inactive as cytokinins showed much less affinity for ribosomes. Pretreatment of ribosomes with 0.5 M-ammonium chloride or Triton X-100 did not decrease the extent of cytokinin binding. Binding appeared to be to the 83S ribosome species. A positive correlation between the extent of binding and the biological effect of various cytokinin analogues was demonstrated. These results are discussed in terms of cytokinin control of growth processes at the ribosomal level.

Animal hormones appear to control both transcriptional and translational processes (Tata, 1968). There is some evidence that the three principal types of phytohormones, auxins, gibberellins and cytokinins, may affect transcription within the nucleus (Letham, 1969). However, certain other observations suggest that cytokinins can also influence growth processes at the ribosomal level. For example, cytokinins are known to maintain the content of ribosomes in excised leaf tissue (Shaw & Manocha, 1965; Srivastava & Arglebe, 1968; Berridge & Ralph, 1969), and they can markedly promote incorporation of amino acids into protein (Parthier & Wollgiehn, 1961; Osborne, 1962; Mothes, 1964; Anderson & Rowan, 1966; Bhattacharyya & Roy, 1969). In a system with plastids *in vitro* this effect is produced in less than 5 min (Davies & Cocking, 1967). The rapidity of this response suggests that the hormone may promote protein synthesis in a very direct way, possibly at the ribosomal level.

Cytokinins occur in nucleotide form in hydrolysates of total tRNA (Hall, 1964; Hall, Robins, Stasivk & Thedford, 1966; Letham & Ralph, 1967; Burrows, Armstrong & Skoog, 1968; Matsubara, Armstrong & Skoog, 1968; Burrows *et al.* 1969). In all cytokinin-containing tRNA species of known sequence the hormone is adjacent to the 3′-end of the anticodon. 6-*N*-(3-Methylbut-2-enyl)-adenosine (isopentenyladenosine) occurs in this position in yeast (Biemann *et al.* 1966) and rat liver (Staehelin, Rogg, Baguley, Ginsberg & Wehrli, 1968) serine tRNA and in yeast tyrosine tRNA (Madison & Kung, 1967). This nucleoside has also been isolated from hydrolysates of yeast cysteine tRNA (Hecht *et al.* 1969) and from hydrolysates of total tRNA from several higher plant tissues (Hall, Csonka, David & McLennan, 1967). The related nucleoside 6-*N*-(3-methylbut-2-enyl)-2-methylthio-adenosine occurs in hydrolysates of *Escherichia coli* serine I and II tRNA (Nishimura, Yamada & Ishikura, 1969), phenylalanine tRNA (Nishimura *et al.* 1969; Barrell & Sanger, 1969), tyrosine tRNA (Harada *et al.* 1968) and Su III tyrosine tRNA (Goodman, Abelson, Landy, Brenner & Smith, 1968; Gefter & Russell, 1969). In the last two mentioned tRNA species the phytohormone is adjacent to the anticodon. It has been suggested that the presence of these unusual bases in tRNA is related to the ability of tRNA species to recognize codons beginning with U (Nishimura *et al.* 1969; Hecht *et al.* 1969; Armstrong *et al.* 1969).

In ribosome-binding assays in response to the codon UAG, *E. coli* tyrosine tRNA containing 6-*N*-(3-methylbut-2-enyl)-2-methylthioadenosine was twice as efficient as tyrosine tRNA containing 6-*N*-(3-methylbut-2-enyl)adenosine and seven times as efficient as unmodified tyrosine tRNA (Gefter & Russell, 1969), suggesting that the modified bases adjacent to anticodons may play some regulatory role in protein synthesis. Since these cytokinins, as their derivatives in tRNA, probably bind to a site in the ribosome, it seems possible that cytokinins

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as free bases might also bind to this same site or to a related site and as a result modify ribosomal processes.

In view of the above observations, an attempt was made to ascertain whether cytokinins bind to plant ribosomes and to assess the significance of any observed binding. In preliminary experiments we preincubated purified chinese-cabbage leaf ribosomes with [8-^{14}C]kinetin (6-furfurylamino-[8-^{14}C]purine) and then recovered them by centrifugation. However, we could not demonstrate binding of kinetin to the recovered ribosomes by this procedure. We report here further experiments demonstrating that certain cytokinins do bind to purified chinese-cabbage leaf ribosomes. Several binding sites appear to be present on ribosomes.

MATERIALS AND METHODS

Preparation of ribosomes

Ribosomes were prepared by grinding chinese-cabbage (*Brassica pekinensis*) leaf tissue with Nirenberg buffer [60mM-KCl, 6mM-mercaptoethanol, 16mM-magnesium acetate and 10mM-tris acetate buffer, pH 7.6] at 4°C. Cell debris was removed by centrifugation at 600*g* for 5min. Smaller fragments and mitochondria were sedimented at 18000*g* for 15min. The supernatant was then centrifuged at 120000*g* for 5min, the supernatant transferred to new tubes and the ribosomes recovered by centrifugation at 225000*g* for 60min. Because of occasional contamination of ribosome pellets with fraction I protein the surface of ribosome pellets was washed with 1ml of buffer. This procedure was effective in removing fraction I protein which is otherwise difficult to remove without gradient centrifugation. The pellet of ribosomes was resuspended in Nirenberg buffer and subjected to a second identical cycle of purification. The final pale-yellow translucent pellet of ribosomes was resuspended in buffer. Sedimentation in a Spinco model E analytical ultracentrifuge showed small peaks of ribosome aggregates sedimenting ahead of the 83S ribosomes. Amounts of individual ribosome species in different preparations were estimated for comparison purposes by measuring the area of the separate peaks in projections of the sedimentation profile (see Table 2).

Characterization of ribosomes

The u.v.-absorption spectrum and the E_{260}/E_{280} ratio was determined for each ribosome preparation. The spectra and ratios obtained (E_{260}/E_{280} 1.85) were typical of ribosomes prepared from other eukaryote species (see Martin, Rolleston, Low & Wool, 1969).

Determinations of the percentage protein in the ribosomes by Folin–Ciocalteu assay and by recovery of total protein after digestion of ribosomes with 5% (w/v) trichloroacetic acid at 90°C for 10min both gave values for total ribosomal protein of 58–60%. This is in good agreement with determinations of the protein composition of plant ribosomes from other sources (Petermann, 1964).

To eliminate further the possibility that the purified ribosomes contained high-molecular-weight extraneous proteins or other components ribosomes (E_{260} 63) in 0.5ml of Nirenberg buffer were applied to a column (26cm × 1.5cm) of Bio-Gel A (exclusion limit 1.5×10^6 daltons) previously pre-equilibrated with buffer. The column was eluted with the same buffer and fractions (2ml) were collected. Quantitative elution of the u.v.-absorbing material occurred as a single peak eluting at the void volume of the column. In contrast, hen's egg white lysozyme (mol.wt. 15000) eluted from the same column as a single peak 22 fractions later (see Fig. 1). Ninhydrin treatment of samples (0.5ml) of the individual 2ml fractions obtained from the Bio-Gel A column was also carried out. To increase the number of terminal α-amino groups in any possible undetected contaminating proteins the individual 0.5ml samples were first incubated with predigested (1h, 37°C) pronase (10μg) at 20°C for 15h, and then treated with ninhydrin. One major ninhydrin-positive peak (detected at 570nm) was found corresponding to those fractions containing the ribosomes (see Fig. 1). The ninhydrin procedure detected ⩾50μg of lysozyme in control experiments. The ribosomes recovered from the major peak of the Bio-Gel A column bound radioactive 6-benzylaminopurine as did unfractionated ribosomes when they were subjected to gel filtration through radioactive Sephadex G-200 columns (see below). Thus gel filtration through Bio-Gel A did not remove a high-molecular-weight kinetin-binding impurity from the ribosomes.

In view of the above observations we feel confident that our chinese-cabbage ribosomes are free from other soluble proteins. Any potential cytokinin-binding protein impurity in the preparations would have to be bound tightly to the ribosomes (*a*) to survive the repeated sedimentation during ribosome preparation, (*b*) to remain associated with the ribosomes on sucrose gradients (see below), and

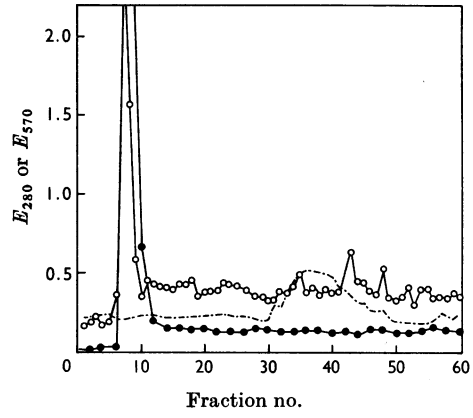

Fig. 1. Elution profile of purified ribosomes from Bio-Gel A columns. Ribosomes or lysozyme were applied to Bio-Gel A columns in Nirenberg buffer (0.5ml) and the columns were eluted with the same buffer. ●, E_{280} of ribosomes; ○, E_{570} of ninhydrin-treated samples; ·–·–·, E_{280} of lysozyme.

(c) to remain associated with the ribosomes during gel filtration through Sephadex G-200 (see below) or Bio-Gel A. Furthermore washing of the ribosomes with 0.5M-NH_4Cl did not prevent their binding cytokinins (see Table 1). This treatment is known to remove many protein factors from *E. coli* ribosomes. Thus it is likely that the binding of cytokinins to ribosomes described below is to the ribosomes themselves rather than to extraneous proteins in the ribosome preparations.

Determination of kinetin binding to ribosomes

Concentration-dependent binding. In initial experiments to detect concentration-dependent binding of [8-^{14}C]kinetin (16.4μCi/μmol) to ribosomes the ultracentrifugation method used by Vasquez (1964) to demonstrate binding of antibiotics to ribosomes was employed. This procedure estimates bound radioactive material by sedimenting separate samples of ribosomes from solutions of decreasing specific radioactivity, in this case decreasing specific radioactivity of [8-^{14}C]kinetin, and increasing concentration. It is necessary to correct for radioactivity in the aqueous volume of the ribosome pellet to determine the amount of radioactive material bound to the sedimented ribosomes. The correction factor is normally determined by extrapolating to zero specific radioactivity the curve of radioactivity associated with the ribosome pellet versus specific radioactivity of the radioactive solution. Because kinetin precipitated at concentrations greater than 44μg/ml it was not possible in our experiments to determine the radioactivity in the aqueous volume of the ribosome pellets in this manner. This difficulty was overcome by sedimenting one sample of the ribosomes from buffer solution containing tritiated water. The volume of water trapped in the ribosome pellet was calculated from the tritium associated with the washed pellet. The amount of [8-^{14}C]kinetin trapped in the aqueous volume of the ribosome pellets was then estimated from the known concentration of [8-^{14}C]kinetin and the volume of water trapped in the pellets. To determine the total [8-^{14}C]kinetin in the ribosome pellets, the pellets were dissolved in 0.7ml of 0.1M-NaOH. Hyamine hydroxide (1M in methanol, 1ml) was added to each tube and the mixture was added to 10ml of dioxan–naphthalene-based scintillant (Carey & Goldstein, 1962). The radioactivity in the samples was determined in a Packard liquid-scintillation spectrometer. Counting efficiency was determined by using an internal standard. The [8-^{14}C]kinetin bound to ribosomes was determined by subtracting the radioactivity in the aqueous volume of the pellets from the total radioactivity in the ribosome pellets. The amount of ribosomes in the ribosomal pellets was determined by assuming that 1mg of ribosomes/ml has an E_{260} of 16. A ribosome molecular weight of 2.7×10^6 was assumed in all calculations for determining numbers of ribosomes.

Sucrose-density-gradient centrifugation. In a second procedure linear (10–40%) sucrose density gradients were prepared with solutions containing 10mM-magnesium acetate, 10mM-KCl, 10mM-tris–HCl buffer, pH7.6, with and without [8-^{14}C]kinetin (0.38μCi/ml; 23μM). Gradients were kept for 90min at 4°C to improve linearity. To 3mg of ribosomes in 0.3ml of Nirenberg buffer, [8-^{14}C]-kinetin (38.3μCi/ml; 3μl) was added and the mixture was kept at 4°C for 10min. Samples (100μl) of the radioactive ribosome solution were then layered on to the sucrose gradients prepared with and without radioactive kinetin. The gradients were centrifuged at 225000*g* for 60min at 4°C, and were then analysed by pumping through an ISCO density gradient fractionator; 22 5-drop fractions were collected. Samples (100μl) from each fraction were used to determine radioactivity. Addition of an internal standard permitted the determination of counting efficiency. In this procedure it was difficult to ensure that the ribosome solutions applied to the gradients had exactly the same radioactivity as the gradient, so that a constant background could be obtained.

Gel filtration. In a third, experimentally simpler, procedure, which was used routinely, columns (0.25cm × 20cm) of Sephadex G-200 were prepared in, and equilibrated with, Nirenberg buffer containing the radioactive compound under investigation. Purified ribosomes were sedimented from Nirenberg buffer containing the radioactive compound, resuspended in the same radioactive buffer, and an appropriate sample was added to the column. The column was eluted with radioactive buffer at 4°C and fractions (30–40) were collected. Samples (70–100μl) from each fraction were used to determine radioactivity, and further samples were diluted to determine the E_{260} values. Fraction sizes in any one experiment were constant but differed between individual experiments because column flow-rates differed. Fractions were normally of 0.1–02ml.

Equilibrium dialysis. In the sucrose-density-gradient and gel-filtration binding assays described above the high radioactivity in the cytokinin solutions necessary to detect cytokinin binding with small amounts of ribosomes led to some oscillation in the measurement of background radioactivity when small portions of the individual fractions were sampled (see Figs. 2 and 3). A more uniform background was subsequently obtained by taking meticulous care when sampling individual fractions. This is reflected in the self-consistent results of further experiments (see Table 3).

Improved 'signal-to-noise' ratios would be possible if more ribosomes could be used to detect binding. Equilibrium dialysis of ribosomes against radioactive cytokinin solutions permits the use of large amounts of ribosomes and this procedure was also used to demonstrate cytokinin binding. Nevertheless, equilibrium dialysis was not considered the method of choice since it did not specifically associate binding with a specific component in the ribosome preparations.

For equilibrium dialysis experiments, ribosomes (E_{260} approx. 50) in Nirenberg buffer (0.25ml) were dialysed for 24h at 4°C against a stirred solution containing the radioactive cytokinin (23μM) in Nirenberg buffer (20ml). Toluene (1 drop) was added to the solution to eliminate bacterial contamination.

Samples (50μl) of the ribosome solution and the diffusate were then assayed for radioactivity and for ribosome concentration (by measurement of E_{260}). In typical experiments the radioactivity in samples of the ribosome solution exceeded that in equal samples of the diffusate by approx. 5000c.p.m. after 24h of dialysis. Preliminary experiments demonstrated that dialysis equilibrium was reached after 8h in well-stirred solutions.

Leaf-disc-expansion assay

Discs (64mm² in area) were removed from young, expanding chinese-cabbage leaves (7–11cm) and floated on test solutions made up in Vickery's solution (Berridge & Ralph, 1969). Equal numbers of discs from each half-leaf were randomly distributed between Petri dishes. Dishes were placed in a light-room (600l/ft²) at 25°C under a 16h photoperiod. After 3 days the discs were washed with water, blotted dry and weighed. Biological effect was measured as a percentage increase in fresh weight as compared with untreated controls.

Leaf-disc-senescence assay

This assay was performed as described by Letham (1967) with discs excised from fully developed chinese-cabbage leaves. Extinctions of 80% ethanol extracts of the aged leaf-discs were determined at 665nm. The difference ($\times 10^3$) between the E_{665} of extract from discs in contact with cytokinin solution and that of extract from discs in contact with water was used as an index of cytokinin-induced chlorophyll retention.

Labelled cytokinins

6-Furfurylamino[8-^{14}C]purine (16.5mCi/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K.; [8-^{14}C]adenosine (50mCi/mmol) was from Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A.; [2,8-$^3H_2$]-adenine (1.74Ci/mmol) was from International Chemical and Nuclear Corp., Irvine, Calif., U.S.A.

6-[G-^{3}H]Benzylaminopurine and 6-[G-^{3}H]morpholinopurine were prepared by the following method. The substituted purines were tritiated at The Radiochemical Centre by catalytic exchange in an aqueous medium. The compounds were stirred with tritiated water and a platinum catalyst for approx. 20h under reflux. The crude products were freed from labile tritium and then purified by preparative t.l.c. on silica gel. The resulting products were further purified by precipitation from an acid aqueous solution (approx. pH2) as a result of pH adjustment to 7. Finally, the products were recrystallized to yield 6-[G-^{3}H]benzylaminopurine (27mCi/mmol) and 6-[G-^{3}H]morpholinopurine (27mCi/mmol).

3-Benzyl[2,8-$^3H_2$]adenine (25mCi/mmol) and 9-benzyl-[2,8-$^3H_2$]adenine (17mCi/mmol) were prepared from [2,8-$^3H_2$]adenine by reaction with benzyl bromide by using modifications of the methods of Montgomery & Thomas (1964) and Leonard, Carraway & Helgeson (1965). In preparing 9-benzyladenine, the reaction mixture after evaporation was subjected to preparative t.l.c. (Merck PF_{254} silica gel; solvent, butan-2-one saturated with water). The eluted product was dissolved in dil. HCl (pH2) and precipitated by adjustment of the pH to 7. By these steps the desired product was separated quantitatively from closely related impurities difficult to remove by crystallization.

RESULTS

Concentration-dependent binding of kinetin to ribosomes. To demonstrate kinetin binding to chinese-cabbage ribosomes we initially used the procedure of Vasquez (1964). The method was slightly modified as outlined in the Materials and Methods section in order to overcome the problem of insolubility of kinetin at concentrations greater than 44μg/ml at 4°C. The results obtained by the modified procedure showed that kinetin bound to ribosomes increased with kinetin concentration over the range of concentrations (0–20μg/ml) where precipitation of kinetin did not occur. At the minimum concentration that was required to produce maximal expansion of floated chinese-cabbage leaf-discs (23μM), 1.5molecules of kinetin were bound per ribosome (Fig. 2). Because of the possibility of spurious or anomalous results in these experiments due to precipitation of kinetin, two alternative procedures were also used to confirm kinetin binding to ribosomes.

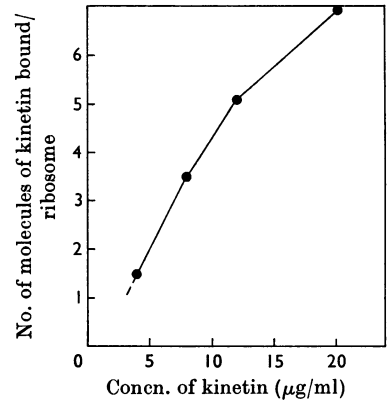

Fig. 2. Concentration-dependent binding of kinetin to ribosomes.

Kinetin binding demonstrated by sucrose-density-gradient centrifugation. Sedimentation of ribosomes preincubated with radioactive kinetin through sucrose density gradients containing the same concentration of radioactive kinetin (0.38μCi/ml; 23μM) showed the presence of additional radioactivity in the region of the ribosomes (Fig. 3*a*). The radioactivity bound to the ribosomes corresponded to 1 molecule of kinetin bound per ribosome. No peak of radioactivity was observed if turnip yellow-mosaic virus was substituted for the ribosomes, confirming that the phenomenon was specifically related to ribosomes in the gradient (Fig. 3*c*). Ribosomes prepared by sedimentation from 0.5M-ammonium chloride bound kinetin to the same extent. No peak of radioactivity was observed in the region of the ribosomes when ribosomes preincubated with radioactive kinetin were sedimented through gradients that did not contain radioactive kinetin (Fig. 3*b*). Thus the

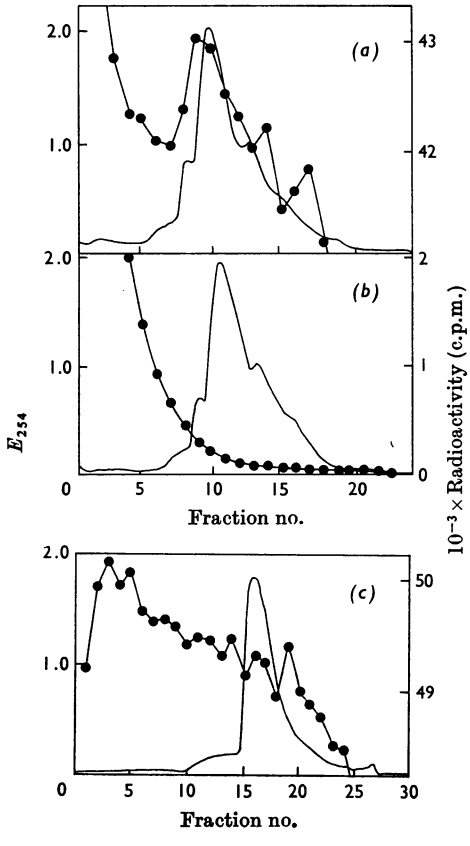

Fig. 3. Sucrose-density-gradient profiles of radioactivity associated with ribosomes preincubated with [8-^{14}C]kinetin. (*a*) Sedimented through a gradient containing [8-^{14}C]-kinetin; (*b*) sedimented through a non-radioactive gradient. The scatter in radioactivity profiles from gradients and columns containing radioactive kinetin is due to the high background radioactivity in gradients containing [8-^{14}C]-kinetin. In most cases it was within sampling error (i.e. <1%). Sedimentation was from left to right. (*c*) Sucrose-density-gradient profiles of turnip yellow-mosaic virus sedimented through a gradient containing [8-^{14}C]kinetin. A solution (0.1 ml) of turnip yellow-mosaic virus (0.94 mg) in Nirenberg buffer containing [8-^{14}C]kinetin (0.383 μCi/μmol; 23 μM) was layered on to a radioactive sucrose density gradient (see Fig. 3*a*). ——, Ribosome (or turnip yellow-mosaic virus) E_{254} profile; ●, radioactivity profile.

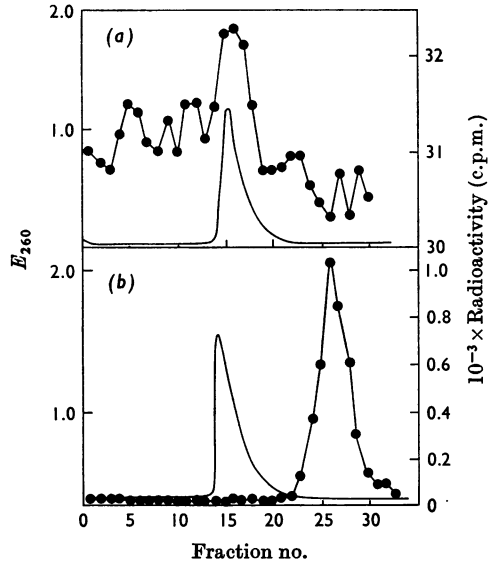

Fig. 4. Elution profile from Sephadex G-200 at 20°C of ribosomes preincubated with [8-^{14}C]kinetin (23 μM). Fraction volume was 0.2 ml. (*a*) Column and eluent containing [8-^{14}C]kinetin. (*b*) Column and eluent not containing [8-^{14}C]kinetin. This column was eluted at 20°C. Much larger peaks of radioactivity were observed in the region of the ribosomes when columns were eluted at 4°C. No peak of radioactivity was detected in the region of the ribosomes at 4 or 20°C if the columns were not pre-equilibrated with [8-^{14}C]kinetin. ●, Radioactivity; ——, E_{260}.

binding appeared to be reversible equilibrium-type binding rather than irreversible tight binding.

Cytokinin binding demonstrated by gel filtration. Gel filtration of ribosomes preincubated with radioactive kinetin through columns of Sephadex G-200 separated the radioactivity from the peak of ribosomal material (Fig. 4*b*). After passage through Sephadex G-200 columns that had previously been equilibrated with the same radioactive kinetin solution (0.38 μCi/ml; 23 μM) in which the ribosomes were suspended, a peak of radioactivity eluted together with the ribosomes (Fig. 4*a*). The radioactivity associated with the peak of ribosomes corresponded to 1 molecule of kinetin bound per ribosome at 4°C. On repetition of this experiment at 20°C 0.5 molecules of kinetin were bound per ribosome, indicating that binding is temperature-dependent (Fig. 4*a*). A more concentrated band of ribosomes was obtained by gel filtration than with sucrose-density-gradient centrifugation. This enabled more accurate determination of radioactivity bound to ribosomes and Sephadex G-200 columns were therefore normally used for further characterization of the nature of cytokinin binding to ribosomes.

Cytokinin-binding demonstrated by equilibrium dialysis. Equilibrium dialysis of purified chinese-cabbage ribosomes against solutions of radioactive cytokinins (23 μM) confirmed that the ribosomes bound 6-benzylaminopurine and kinetin to a greater extent than 9-benzyladenine (Table 1). The order of increasing binding (6-benzylaminopurine > kinetin > 9-benzyladenine) was generally similar to

Table 1. *Cytokinin-binding to ribosomes*

Test compound	Initial sp. radioactivity of diffusate (μCi/μmol)	No. of molecules bound/ribosome*
6-Benzylaminopurine	27	1.00
Kinetin	16.5	0.69
9-Benzyladenine	17	0.52

* Determined by equilibrium dialysis.

Table 2. *Kinetin-binding to ribosomes prepared by various techniques*

Expt. no.	Treatment	% of each ribosome species in preparation†				No. of kinetin molecules bound/ribosome
		30–60S	68S	83S	120S	
A 1	NB*	1.2	11.2	78.8	8.8	1.01
2	NB+NH_4Cl	5.4	12.5	76.3	5.8	0.94
3	NB+1 mM-$MgCl_2$	3.0	0	90.0	7.0	2.50
4	NB+1% Triton X-100	0	32.8	51.4	15.8	0.52
B 5	NB	0	4.3	90.6	5.1	1.05
	NB (Washed with Triton X-100)	0	4.3	90.6	5.1	0.99

* Abbreviation: NB, Nirenberg buffer.
† Estimated from areas of sedimentation profiles.

that detected by the other procedures (see Tables 2 and 3).

Possible factors influencing kinetin binding. Since kinetin might bind to protein factors associated with ribosomes alternative methods that remove protein factors from bacterial ribosomes were used to prepare chinese-cabbage ribosomes. After extraction into Nirenberg buffer and sedimentation in the ultracentrifuge, ribosomes were resuspended in Nirenberg buffer containing 0.5 M-ammonium chloride and twice recovered by centrifugation from 0.5 M-ammonium chloride. The resulting washed ribosomes were finally resuspended in Nirenberg buffer containing radioactive kinetin (0.38 μCi/ml; 23 μM) and the bound kinetin was determined by gel filtration as described above. Ribosomes were also prepared in (*a*) Nirenberg buffer, (*b*) Nirenberg buffer containing 1% Triton X-100, and (*c*) modified Nirenberg buffer containing only 1 mM-magnesium chloride. Each preparation was subjected to three purification cycles in the ultracentrifuge. After this treatment ribosomes prepared as in (*a*) and (*b*) were resuspended in Nirenberg buffer containing radioactive kinetin (0.38 μCi/ml; 23 μM) and those prepared as in (*c*) were resuspended in buffer containing 1 mM-magnesium chloride and radioactive kinetin. Samples of the resuspended ribosomes (E_{260} 20) were then passed through Sephadex G-200 columns that had been pre-equilibrated with radioactive kinetin in the appropriate buffers. Other samples were examined in the Spinco model E analytical ultracentrifuge to determine the ratios of the various ribosome species in each preparation. Table 2 compares the distribution of ribosome species in each preparation with the number of molecules of kinetin bound per ribosome. It is apparent that there is a correlation between the kinetin bound per ribosome and the proportion of 83S ribosomes in the individual ribosome preparations (Table 2, lines 1, 2 and 4). At low magnesium concentrations increased binding of kinetin was observed. Treatment of ribosomes with 0.5 M-ammonium chloride or 1% Triton X-100 did not alter their ability to bind kinetin, suggesting that the binding observed was not to easily removable protein associated with the ribosomes.

Specificity and significance of cytokinin binding. In an attempt to assess the functional significance of cytokinin binding to ribosomes, the biological activities and binding affinities of a series of substituted adenines were determined. A correlation between these properties would imply that the binding was of functional significance.

The influence of structure on the ability of substituted adenines to promote expansion of chinese-cabbage leaf-discs is not known. Therefore a series of compounds were first synthesized and tested for activity in the leaf-disc assay system. Certain structure–activity relationships observed in the commonly used tobacco-pith tissue-culture assay (Skoog *et al.* 1967) were also found to apply to this leaf-disc expansion test. Thus 6-*N*-substituted

adenines were active whereas 3- and 9-substituted adenines exhibited negligible activity; substitution of the 3 position of a 6-*N*-substituted adenine diminished activity; addition of a methyl group to the N^6-position of an active compound depressed activity. However certain minor differences in response were observed between the two systems. Thus, in the tobacco-pith test, amides such as 6-benzamidopurine are reported to be inactive (Skoog *et al.* 1967) whereas, in the chinese-cabbage leaf-disc assay, 6-benzamidopurine and 6-butyryl-aminopurine evoked a response similar to that of 6-benzylaminopurine. In the tobacco-pith and certain other growth tests, the naturally occurring cytokinins, zeatin and 6-(3-methylbut-2-enyl-amino)purine, have been found to be more active than synthetic cytokinins such as 6-benzylamino-purine and kinetin (Skoog *et al.* 1967; Letham, 1968). In the chinese-cabbage leaf-disc assay, however, the converse applies.

To establish the specificity of binding of cyto-kinins to ribosomes radioactive adenine, adenosine, 3-benzyladenine, 6-benzylaminopurine, 9-benzyl-adenine and 6-morpholinopurine were tested for binding to chinese-cabbage ribosomes by using the gel-filtration procedure. Fresh ribosomes were prepared for each experiment. Table 3 lists the results of four separate binding experiments and Table 4 indicates the cytokinin activities of the various compounds and correlates these activities with binding. 6-Benzylaminopurine, the compound with greatest activity in both the leaf-disc expansion and leaf-disc senescence assays, showed greatest binding (1.35 molecules per ribosome). Activities of the various compounds in both assays are positively and significantly correlated with capa-city to bind to ribosomes. The compound that exhibited greatest divergence from the general correlative trend was 6-morpholinopurine which did not bind appreciably to ribosomes but did show some activity in the leaf-disc expansion assay at 23 μM. This activity exhibited greater variability

Table 3. *Specificity of cytokinin-binding to ribosomes*

Expt. no.	Test compound	No. of molecules bound/ribosome
1	6-Benzylaminopurine	1.38
	Adenosine	0.34
2	6-Benzylaminopurine	1.30
	9-Benzyladenine	0.53
	6-Morpholinopurine	<0.10
	6-Benzylaminopurine (*S. oligorrhiza*)	1.08
3	6-Benzylaminopurine	1.33
	Adenine	<0.10
	3-Benzyladenine	0.51
4	6-Benzylaminopurine	1.32
	6-Benzylaminopurine+ 3-benzyladenine	0.96
	6-Benzylaminopurine+ puromycin	1.06
	6-Benzylaminopurine+ tRNA	1.16

Table 4. *Comparison of the activities of purine derivatives in causing expansion and retarding senescence of chinese-cabbage leaf-discs, and correlation of these activities with degree of ribosome binding*

Test compound	Disc expansion (% increase in fresh weight compared with control)		Index of cytokinin-induced chlorophyll retention	No. of molecules bound/ribosome
Concentration ...	23 μM	2.3 μM	2 μM	
Adenosine	1.7	0.5	4	0.34
Adenine	5.8	0.0	0	<0.10
3-Benzyladenine	8.9	6.8	0	0.51
9-Benzyladenine	9.3	7.1	3	0.53
6-Morpholinopurine	17.0	4.6	7	<0.10
Kinetin	23.8	18.7	36	1.00 ± 0.05
6-Benzylaminopurine	29.0	23.4	52	1.35 ± 0.05

Correlation	Correlation coefficient*	Level of significance (%)
Between disc expansion (23 μM) and no. of molecules bound/ribosome	0.761†	5
Between disc expansion (2.3 μM) and no. of molecules bound/ribosome	0.947	1
Between index of cytokinin-induced chlorophyll retention and no. of molecules bound/ribosome	0.896	1

* Computed as detailed by Snedecor (1956).

† In subsequent repeated bioassays, 6-morpholinopurine exhibited lower activity, and the disc-expansion data correlated better with ribosomal binding.

than that of the other cytokinins and ranged from 30 to 59% of that of 6-benzylaminopurine. The activity of 6-morpholinopurine in the experiment of Table 3 was the maximum observed with this compound. The cytokinin activity of the compound was highest over a relatively narrow concentration range and at 50 μg/ml it caused necrosis of leaf tissue.

Slight inhibition of binding of 6-benzylaminopurine (5 μg/ml) to chinese-cabbage ribosomes was observed when binding assays were carried out in the presence of 3-benzyladenine (5 μg/ml), puromycin (20 μg/ml) or purified chinese-cabbage tRNA (15 μg/ml), suggesting that each of these compounds might compete for or modify the cytokinin binding site or sites (Table 3, expt. 4).

Binding of 1.08 molecules of 6-benzylaminopurine (23 μM) per ribosome was also detected with *Spirodela oligorrhiza* ribosomes indicating that the phenomenon is not unique to chinese-cabbage ribosomes (Table 3). We have confirmed that 6-benzylaminopurine can substitute for kinetin in supporting the growth of *S. oligorrhiza* in the dark (P. J. McCombs & R. K. Ralph, unpublished work).

Adenosine 3′:5′-cyclic phosphate. Because many animal hormones appear to exert their effects by altering the concentrations of adenosine 3′:5′-cyclic phosphate in target tissues we examined the effects of adenosine 3′:5′-cyclic phosphate on floated leaf-discs. There was no detectable effect on the fresh weight of discs floated for 3 days on 12.7 μM adenosine 3′:5′-cyclic phosphate compared with controls. In contrast, when discs were floated on *NO*-dibutyryladenosine 3′:5′-cyclic phosphate (23 μM) a marked increase in fresh weight occurred comparable with that seen with 6-benzylaminopurine or kinetin. However, we were unable to conclude that this increase resulted from the action of adenosine 3′:5′-cyclic phosphate since 6-butyrylaminopurine was found to be as active as 6-benzylaminopurine in this system and we could not exclude the possibility that 6-butyrylaminopurine could have been produced *in vivo* by degradation of *NO*-dibutyryladenosine 3′:5′-cyclic phosphate. In animal systems *NO*-dibutyryladenosine 3′:5′-cyclic phosphate appears to enter cells more readily than does adenosine 3′:5′-cyclic phosphate.

DISCUSSION

In other systems binding of small molecules to ribosomes has been shown to have functional significance. For example, chloramphenicol binds reversibly to *E. coli* ribosomes and interferes with protein synthesis. Streptomycin, tetracycline, actidione, puromycin and many other drugs bind to ribosomes and modify ribosomal processes. Even adenosine 3′:5′-cyclic phosphate has been shown to bind to microsomes (Garen, 1969). Frequently this type of binding appears to be reversible and concentration-dependent. From the present results we conclude that the cytokinins kinetin and 6-benzylaminopurine can bind to chinese-cabbage leaf ribosomes, 1.00 and 1.35 molecules being bound per ribosome with the compounds at 23 μM. That this binding may be functionally significant is suggested first by the fact that 23 μM-kinetin or 6-benzylaminopurine is the minimum amount required in the medium to produce maximum expansion of floated chinese-cabbage leaf-discs and secondly by the positive correlation demonstrated between binding and cytokinin activity (Tables 3 and 4).

Chinese-cabbage ribosomes appear to have multiple binding sites for kinetin or 6-benzylaminopurine. The significance of this as regards their possible biological function remains to be determined. However, above a certain concentration (23 μM) floated chinese-cabbage leaf-discs show no further response to additional kinetin, suggesting that binding of one kinetin molecule per ribosome may be sufficient to elicit the full biological effect. We could not demonstrate a limit to cytokinin binding to ribosomes in these experiments because of the insolubility of all of the cytokinins at higher concentrations.

Sucrose-density-gradient centrifugation failed to separate cleanly the 68S and 83S ribosomes so that kinetin-binding to a specific ribosome species was not directly demonstrable by using this technique (Fig. 3). Nevertheless, the coincidence of the peak of bound radioactive kinetin with the peak of 83S ribosomes suggested that these ribosomes were the major species binding kinetin. More convincing evidence that 83S rather than 68S ribosomes bind kinetin may be seen in Table 3. Variation of the proportion of 68S ribosomes present in the different ribosome preparations was not accompanied by equivalent variations in the number of kinetin molecules bound per ribosome. In fact the reverse was true, the greatest kinetin binding per ribosome occurring in preparations rich in 83S ribosomes (Table 2). It is unlikely that the presence of small amounts of ribosomal subunits and dimers could significantly influence these results. However, we are attempting to isolate enough pure 68S ribosomes to demonstrate that these do not bind cytokinins. At present we have no clear explanation why the preparation of ribosomes (Table 2, expt. 4) contained such a high proportion of 68S ribosomes. It is noteworthy that low Mg^{2+} concentrations preferentially dissociate chloroplast 68S ribosomes.

Although the experiments with ammonium chloride-washed ribosomes were carried out to determine whether binding occurs to protein

factors on ribosomes, failure to detect any effect of ammonium chloride is not conclusive since the effect of 0.5M-ammonium chloride on plant ribosomes is not known. The procedure is effective in removing certain protein factors from bacterial ribosomes. Binding does appear to be influenced by Mg^{2+} concentration and by temperature, both factors known to affect ribosomes.

In other experiments (M. V. Berridge, R. K. Ralph & D. S. Letham, unpublished work) designed to define the functional significance of kinetin binding to ribosomes we have demonstrated that, compared with untreated controls, kinetin (23μM) increases the incorporation of [^{35}S]sulphate into soluble proteins of floated chinese-cabbage leaf-discs within 1.5–2h. We have also developed systems from chinese-cabbage leaves synthesizing RNA and protein *in vitro* similar to those prepared from spinach leaves by Spencer (1965) and Spencer & Whitfeld (1967). These systems contain primarily chloroplasts and nuclei. Added kinetin failed to affect either RNA or protein synthesis in such systems. Elimination of the chloroplasts by Triton-X-100 treatment gave nuclei still active for RNA synthesis. However, added kinetin failed to affect RNA synthesis with these nuclei. Since protein synthesis in plant extracts containing nuclei and chloroplasts probably occurs principally on ribosomes that were already engaged in protein synthesis *in vivo* these experiments have not excluded the possibility that kinetin can modify initiation of protein synthesis *in vivo* by binding to ribosomes. Further, protein synthesis in these extracts is thought to occur mostly in chloroplasts (Spencer, 1965) that contain 68S ribosomes (Clark, 1964) and our evidence suggests that 68S ribosomes do not bind kinetin. As yet we have not been successful in developing an active protein-synthesizing system from chinese-cabbage containing free 83S ribosomes and responsive to added mRNA. Such a system would be most useful for investigating the functional significance of kinetin-binding to chinese-cabbage leaf 83S ribosomes.

Finally, growth evoked by cytokinins in chinese-cabbage leaf-discs is probably a consequence of cell enlargement (Miller, 1956; Berridge & Ralph, 1969) whereas growth elicited by these hormones in tobacco-pith tissue involves induction of cell division. However, both systems show similar activity–structure relationships. This suggests that cytokinins may function at the same site in both systems. Hence cytokinin-binding to ribosomes may be of functional significance not only in promotion of cell enlargement but also in induction of cell division. Whether the binding sites on ribosomes are normally responsive to the cytokinin moiety present in tRNA or to free cytokinin *in vivo* is still an intriguing question.

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