Biochem. J. (1972) **129**, 403–417
Printed in Great Britain

Protein, Nucleic Acid and Starch Metabolism in the Duckweed, *Spirodela oligorrhiza*, Treated with Cytokinins

By P. J. A. McCOMBS and R. K. RALPH
Department of Cell Biology, University of Auckland, Auckland, New Zealand

(*Received* 5 *April* 1972)

Bacteria-free cultures of *Spirodela oligorrhiza* continue to increase in frond number for 2 to 3 days after transfer to darkness. There is then no further increase in frond number for 3 to 4 weeks, although DNA, RNA and protein synthesis continue at decreased rates and starch accumulates in the plants. We refer to such 'non-growing' plants in darkness as dormant. Adding kinetin to dormant *Spirodela* initiated increased DNA, RNA and protein synthesis within 1h, although new fronds were not detected until 24h after the addition of kinetin. The frond number then continued to increase. Starch accumulated in dormant plants. Accumulation of starch appeared to be a consequence of inhibition of growth rather than the converse. No evidence was obtained for a block in [^{14}C]glucose metabolism that might explain the lack of growth in darkness in the absence of kinetin. In darkness, more ribosomes were membrane-bound in dormant *Spirodela* than in *Spirodela* growing with kinetin. Similarities between the response of *Spirodela* to darkness, stringent control in bacteria and pleiotypic controls in animal cells are discussed. It is suggested that all three processes are ultimately controlled by specific protein kinases that are individually sensitive to different effectors.

Cytokinins stimulate the incorporation of amino acids into plant proteins (Osborne, 1962; Mothes, 1964; Anderson & Rowan, 1966; Davies & Cocking, 1967; Battacharyya & Roy, 1969; Mizrahi *et al.*, 1970; Richmond *et al.*, 1971). The possibility that they act directly on protein synthesis has been suggested, but is far from proven (Berridge *et al.*, 1970; also see L. Dure and G. L. Farkas, both cited in the Report on the Hungarian Academy of Sciences Conference, 1971). In many situations increases in protein synthesis with cytokinins appear to be preceded or accompanied by increased RNA synthesis (Sugiura *et al.*, 1962; Letham, 1967*b*; Filner *et al.*, 1969; Rijven & Parkash, 1971).

Numerous reports have associated cytokinin action with nucleic acid metabolism (see reviews by Oota, 1964; Helgeson, 1968; Trewavas, 1968; Key, 1969). For example, cytokinins maintain or induce accumulation of RNA in many tissues (Guttman, 1957; Osborne, 1962; Jensen *et al.*, 1964; Berridge & Ralph, 1969; Paranjothy & Wareing, 1971). Cytokinins may also be incorporated into plant tRNA (Fox & Chen, 1967, 1968; Richmond *et al.*, 1970; Burrows *et al.*, 1971). Reports that cytokinins stimulate RNA synthesis in isolated nuclei (Matthysee & Abrams, 1970) have not yet been adequately confirmed. Our attempts to repeat these studies failed to show any effect of kinetin on RNA synthesis in isolated nuclei *in vitro*.

We report here a detailed study of the effect of cytokinins on protein, nucleic acid, starch and glucose metabolism in sterile cultures of *Spirodela oligorrhiza* in darkness. *Spirodela* has a requirement for intermittent red light, or cytokinins, to grow continuously after transfer to darkness. When transferred to darkness on medium without cytokinins, growth ceases and the plants remain dormant for 3 to 4 weeks (Gorham, 1950; Letham, 1967*a*) before growth resumes spontaneously. Addition of kinetin at any time during the 4 weeks of dormancy initiates growth immediately (P. J. A. McCombs & R. K. Ralph, unpublished work). The system has many advantages for investigating cytokinin action. We emphasize similarities between our results and observations on stringent control in bacteria and pleiotypic control in animal cells.

Experimental

Chemicals

The sources of several of the compounds used in this investigation are given below.

Chloramphenicol, Parke Davis and Co., Detroit, Mich., U.S.A.; actidione, K and K Labs., Hollywood, Calif., U.S.A.; kinetin, Calbiochem, Los Angeles, Calif., U.S.A.; 5-fluorodeoxyuridine, Hoffman–La Roche, Basle, Switzerland; [^{3}H]uridine (20Ci/mmol), Schwarz BioResearch, New York, U.S.A.; [^{3}H]thymidine (20.1 Ci/mmol), [^{35}S]sulphate (carrier-free), [^{32}P]P_i (carrier-free) and [^{14}C]glucose (300mCi/mmol), The Radiochemical Centre, Amersham, Bucks., U.K.

Methods

Measurement of radioactivity. The ^3H and ^{14}C radioactivity associated with glass-fibre filters was measured with a Packard liquid-scintillation spectrometer (model no. 3003) using a toluene-based scintillant (Calfluor II); otherwise radioactivity was measured on planchettes with a Nuclear–Chicago low-background gas-flow radiation monitor (model no. 1043).

Culture of Spirodela oligorrhiza. Unless otherwise stated, metabolic studies were performed with bacteria-free cultures of *Spirodela* growing on 100ml of medium in 500-ml Erlenmeyer flasks. Flasks in darkness were in light-tight boxes. Stock light-grown cultures were grown under two Osram 40W daylight fluorescent tubes at a light-intensity of 3550lx. In individual experiments three different media were used. The basal medium used for most experiments was as follows: NH_4NO_3 (2mM), $(NH_4)_2SO_4$ (3mM), $Ca(NO_3)_2$ (2mM), KNO_3 (2mM), $MgSO_4$ (2mM), K_2HPO_4 (1mM), KCl (0.88mM), H_3BO_3 (46μM), $MnSO_4$ (8.9μM), $ZnSO_4$ (7.6μM), $CuSO_4$ (3.2μM), $(NH_4)_2Mo_7O_{24}$ (3.2μM), EDTA, Fe salt (5μM), glucose (1%, w/v). Before autoclaving, the pH was adjusted to 6.4 with H_2SO_4 and an excess of solid calcium orthophosphate was added to maintain the pH during growth between 5.3 and 5.6.

For experiments with [^{35}S]sulphate, low-sulphate medium was used of the following composition: NH_4NO_3 (3.5mM), NH_4Cl (1.5mM), $Ca(NO_3)_2$ (2mM), KNO_3 (2mM), $MgCl_2$ (2mM), K_2HPO_4 (1mM), KCl (0.88mM) and trace elements as above. The adjustment of pH was with HCl. For experiments with [^{32}P]P_i a low-phosphate medium used was: $MgSO_4$ (2mM), K_2SO_4 (1mM), K_2HPO_4 (1mM), $Ca(NO_3)_2$ (2mM), and trace elements as above. Adjustment of pH was with H_2SO_4. Media were sterilized at 15lb/in^2 (100kN/m^2) for 30min.

Addition or withdrawal of *Spirodela* from flasks in darkness was carried out in a dark-room in a sterile hood with dim phytochrome-inactive light from a filter. Wire loops were sterilized in alcohol and acetone.

Treatment of samples. Before homogenization for determination of various constituents, samples of *Spirodela* taken from growth flasks were washed extensively in ice-cold 0.1% sodium dodecyl sulphate, and then with cold water, blotted dry and weighed. In experiments with radioactive sulphate, phosphate or glucose, the wash solutions also contained respectively 0.1M-sodium sulphate, 0.1M-disodium phosphate or 1% glucose. Samples were ground in a 10-ml Kontes Duall glass tissue-homogenizer.

Determination of soluble protein. Samples of *Spirodela* (approx. 200mg) were homogenized at 0°C with 3ml of 10mM-tris–HCl–10mM-NaCl–5mM-EDTA–10mM-$MgCl_2$ buffer, pH7.6, and centrifuged at 18000*g* for 15min. Protein in the 18000*g* supernatant was determined by the Folin–Lowry method (Lowry *et al.*, 1951).

Determination of radioactivity in protein. Samples (approx. 200mg) of *Spirodela* were homogenized at 0°C with 3ml of 10mM-tris–HCl–10mM-NaCl–5mM-EDTA–10mM-$MgCl_2$ buffer, pH7.6, and centrifuged at 18000*g* for 15min. Portions (1ml) of the supernatant were precipitated by adding trichloroacetic acid (final concentration 5%), and the pellet was resuspended in 5% (w/v) trichloroacetic acid. These suspensions were heated at 100°C for 7.5min. The remaining precipitates were collected on Whatman GF/C glass-fibre filters and the associated radioactivity was determined.

Extraction of nucleic acid. Nucleic acid was extracted from *Spirodela* by homogenizing with 3ml of 0.5% sodium dodecyl sulphate, 0.5% disodium naphthalenedisulphonate, 1% (w/v) polyvinylpyrrolidone and 2ml of aq. 80% (w/v) phenol containing 0.1% (w/v) 8-hydroxyquinoline, or with 3ml of 6% (w/v) sodium *p*-aminosalicylate, 1% (w/v) polyvinylpyrrolidone and 2ml of aq. 80% phenol containing 0.1% (w/v) 8-hydroxyquinoline. The homogenates were centrifuged at 18000*g* for 15min and the aqueous phase carefully removed. For some purposes phenol was removed from the aqueous phase by extraction with ether.

Determination of RNA. RNA extracted from *Spirodela* (200mg) with 6% sodium *p*-aminosalicylate–1% polyvinylpyrrolidone was determined with orcinol (Mejbaum, 1939) after removal of phenol by ether extraction.

The total nucleic acid content of *Spirodela* (2g) was determined by using the extraction procedure of Matthews (1958).

Determination of DNA. DNA extracted from *Spirodela* (200mg) into 6% sodium *p*-aminosalicylate–1% polyvinylpyrrolidone was determined by the improved Dische method (Giles & Myers, 1965) after removal of phenol with ether. Salmon sperm DNA was used as standard.

Determination of radioactivity in nucleic acid. RNA. [^3H]Uridine radioactivity incorporated into nucleic acid was determined by extracting *Spirodela* with 0.5% sodium dodecyl sulphate, 0.5% disodium naphthalenedisulphonate, 1% polyvinylpyrrolidone and phenol. After centrifugation at 15000*g* for 15min the nucleic acid in 1ml portions of the aqueous supernatant was precipitated with trichloroacetic acid (final concentration 5%). Alkali-labile radioactivity (65°C, 90min, 1M-NaOH) in the precipitate was recorded as RNA. Previous experiments confirmed that this treatment completely solubilized RNA and that the material solubilized was RNA, since it was also ribonuclease-labile.

DNA. Alkali-stable radioactivity in extracts prepared as described above from *Spirodela* grown with [^3H]thymidine was recorded as DNA. Previous

experiments confirmed that this material was hydrolysed by pancreatic deoxyribonuclease I.

Extraction and determination of starch. *Spirodela* was homogenized in 3ml of buffer [0.01M-tris–HCl (pH7.6)–0.01M-$MgCl_2$] and the homogenizer was rinsed with a further 2ml of the buffer. The homogenate was centrifuged at 12000*g* for 10min and the pellet extracted twice with 10ml of the buffer containing 2% Triton X-100 (to free starch grains from chloroplasts). After re-centrifuging at 12000*g* for 10min, the pellet was suspended in 2ml of the buffer and incubated with 10μg of pancreatic deoxyribonuclease I for 10min at 20°C. The remaining insoluble material was dehydrated with 95% (w/v) ethanol and twice with acetone. Starch in the dry residue was hydrolysed with 50% (v/v) $HClO_4$ and the resulting sugars were determined by the anthrone method (McCready *et al.*, 1950).

Determination of radioactivity in starch. Starch in residues prepared as described above was hydrolysed with 1.3ml of 50% (v/v) $HClO_4$ for 15min at 20°C. Insoluble material was removed by centrifugation at 10000*g* and the pellet was extracted twice with water (90°C for 10min, and 20°C for 10min). The pooled supernatants were adjusted to pH7 with 5M-KOH, the resulting precipitate was removed by centrifugation, and the hydrolysate was dried under vacuum. The residue was dissolved in water (1ml) and radioactivity in portions of this solution determined.

Effect of kinetin on protein synthesis. Light-grown *Spirodela* was transferred into two flasks containing low-sulphate medium. One flask contained kinetin (250ng/ml). To allow for growth about 20% less of the *Spirodela* was placed in the flask containing kinetin. After 4 days in darkness, by which time growth had ceased in the absence of kinetin, 1ml of 10mM-$(NH_4)_2SO_4$ containing 500μCi of [^{35}S]sulphate (carrier-free) was added to each flask under dim green light and the flasks were returned to darkness. The uptake and incorporation of [^{35}S]sulphate into protein in each culture in darkness was followed over 72h. Samples removed from the cultures were thoroughly washed, and the ^{35}S incorporated into 18000*g* 'soluble' and 'pellet' proteins was determined (see above). The radioactivity associated with 100μl of the 18000*g* supernatant was also determined and the total [^{35}S]sulphate taken up was calculated as the sum of that in the pellet and that in the total supernatant.

Determination of the specific radioactivity of sulphate in Spirodela. Light-grown *Spirodela* was transferred into two flasks containing low-sulphate medium. One flask contained kinetin (250ng/ml) and about 20% less of the *Spirodela* to allow for growth. After 4 days in darkness, 1ml of 10mM-$(NH_4)_2SO_4$ containing 500μCi of [^{35}S]sulphate (carrier-free) was added to each flask. Samples of *Spirodela* were withdrawn at the time of [^{35}S]sulphate addition and daily thereafter. The samples were washed with ice-cold detergent containing 0.1M-sodium sulphate, then extensively with ice-cold water to ensure removal of residual sulphate. After being weighed, the individual samples were homogenized in 2ml of water and 1ml of aq. 80% phenol, then the homogenates were centrifuged at 18000*g* for 10min. A separate experiment confirmed that [^{35}S]sulphate distributes >90% in the aqueous phase in such a phenol extraction. Sulphate in the aqueous phase was determined by reduction to H_2S (Johnson & Ulrich, 1959). At the end of the assay a sample (50μl) of the spent reagent remaining in the reaction flask was dried on to a planchette and the associated radioactivity was measured to ensure that reduction was complete.

Effect of inhibitors on protein synthesis in Spirodela. Five flasks were prepared, each containing *Spirodela* on 20ml of low-sulphate medium. One flask contained kinetin (250ng/ml), one chloramphenicol (20μg/ml), one actidione (1μg/ml) and one both chloramphenicol (20μg/ml) and actidione (1μg/ml). All five flasks were placed in darkness. After 4 days, 200μCi of [^{35}S]sulphate (carrier-free) was added to each flask in 1ml of low-sulphate medium. After a further 24h in darkness, the *Spirodela* was removed, washed, dried and weighed. Each sample was homogenized and the radioactivity associated with 18000*g* 'soluble' and 'pellet' protein was determined (see above). Preliminary experiments showed that 20μg of chloramphenicol/ml and 1μg of actidione/ml were the lowest concentrations of the drugs that completely inhibited growth in the light.

Estimation of time required for stimulation of protein synthesis by kinetin. Light-grown *Spirodela* was transferred to three flasks containing low-sulphate medium. One flask also contained kinetin (250ng/ml). To allow for growth about 20% less of the *Spirodela* was added to the flask containing kinetin. After 4 days in darkness 1ml of 10mM-$(NH_4)_2SO_4$ containing 1mCi of [^{35}S]sulphate (carrier-free) was added to each flask. After a further 14h in darkness samples (approx. 200mg) of *Spirodela* were withdrawn from the individual flasks at intervals over the next 16h. At 18h after the addition of [^{35}S]sulphate, 25μg of kinetin in 1ml of low-sulphate medium was added to one of the flasks lacking kinetin and the sampling time-course was continued. The samples from each time-point were used to determine radioactivity in 'soluble' protein and to follow the [^{35}S]sulphate uptake (see above).

Effect of kinetin on RNA synthesis. Two flasks containing *Spirodela* were placed in darkness. One flask contained kinetin (250ng/ml) and about 20% less of the *Spirodela*. After 4 days in darkness, 10μCi of [^{3}H]uridine (sp. radioactivity 20Ci/mmol) in 1ml of water was added to each flask. At regular intervals over 24h samples of *Spirodela* were withdrawn and the radioactivity incorporated into RNA was

determined. After phenol extraction the total radioactivity associated with 100μl of the aqueous phase was also determined.

Estimation of time required for stimulation of RNA synthesis by kinetin. Light-grown *Spirodela* was transferred to three flasks containing 100ml of basal medium. One flask contained kinetin (250ng/ml) and about 20% less of the *Spirodela*. After 4 days in darkness 30μCi of [^{3}H]uridine (sp. radioactivity 20Ci/mmol) in 1 ml of medium was added to each flask. Beginning 8h later, samples (approx. 200mg) of *Spirodela* were withdrawn at intervals. At 14h after the addition of [^{3}H]uridine, kinetin (25μg) in 1ml of medium was added to one of the flasks lacking kinetin and the sampling time-course was continued. The total radioactivity and radioactivity in RNA in each sample were determined.

Synthesis and turnover of RNA in growing and non-growing Spirodela. Light-grown *Spirodela* was transferred to a flask containing 200μCi of [^{3}H]uridine (sp. radioactivity 20Ci/mmol) in 100ml of basal medium. The flask was placed in darkness for 4 days and then the contents were divided and transferred, after a rinse in sterile water, to two flasks each containing 100ml of basal medium with 10μM-uridine. One flask also contained kinetin (250ng/ml). At intervals over 25 days samples of *Spirodela* (approx. 200mg) were withdrawn from each flask and the radioactivity associated with RNA in the samples was determined.

Estimation of free and membrane-bound ribosomes. *Spirodela* in 100ml of low-phosphate medium was pre-labelled with carrier-free [^{32}P]P_i (1mCi) in the light for 3 days. The plants were then transferred to two flasks with medium containing excess of calcium orthophosphate. One flask contained kinetin (250ng/ml). The flasks were placed in darkness for 4 days. After 4 days 1g of *Spirodela* from each culture was homogenized in 3ml of buffer [0.01M-tris–HCl (pH7.6)–0.01M-$MgCl_2$]. The homogenates were centrifuged at 18000*g* for 15min to sediment membrane-bound ribosomes and the resulting supernatants centrifuged at 180000*g* for 2h to sediment free ribosomes.

Nucleic acids were extracted from each pellet and ^{32}P radioactivity present in acid-precipitable, alkali-labile RNA was determined.

Uptake and incorporation of [^{3}H]thymidine into DNA. Two flasks containing *Spirodela* were placed in darkness. One flask contained kinetin (250ng/ml) and about 20% less of the *Spirodela*. After 4 days in darkness, 10μCi of [^{3}H]thymidine (sp. radioactivity 20.1Ci/mmol) was added to each flask. At intervals over 24h samples of *Spirodela* were withdrawn and the radioactivity incorporated into DNA was determined after phenol extraction. The total radioactivity associated with 100μl of the aqueous phase of the extraction was also determined.

Estimation of time required for stimulation of DNA synthesis by kinetin. Light-grown *Spirodela* was placed into darkness in two flasks. After 4 days 1ml of medium containing 100μCi of [^{3}H]thymidine (sp. radioactivity 20.1Ci/mmol) was added to each flask. Beginning 8h later, samples (approx. 200mg) of *Spirodela* were withdrawn at intervals. At 14h after [^{3}H]thymidine addition, 25μg of kinetin in 1ml of water was added to one flask and the sampling time-course continued. The total radioactivity and radioactivity in DNA in each sample was determined.

Uptake and incorporation of [^{14}C]glucose by Spirodela in darkness. Light-grown *Spirodela* was transferred, via a rinse in sterile water, to two flasks containing 100ml of medium with 0.1% glucose. One flask contained kinetin (250ng/ml). Both flasks were placed in darkness. After 4 days each culture was transferred, after a wash in sterile water, to a flask containing 50ml of fresh medium without glucose. The plants growing on kinetin were transferred to a flask containing kinetin (250ng/ml). [^{14}C]Glucose (25μCi; sp. radioactivity 300mCi/mmol) was then added to each flask and the flasks returned to darkness. Samples of *Spirodela* were removed from the flasks at intervals over 3 days, rinsed, dried, weighed and homogenized in 2.5ml of 0.01M-tris–HCl–0.01M-NaCl – 5mM-EDTA – 0.01M-$MgCl_2$ buffer, pH7.5. The homogenates were centrifuged at 1000*g* for 5min. The pellets were discarded and the supernatants centrifuged at 18000*g* for 15min. The radioactivity in 50μl of each supernatant was then determined. Each 18000*g* pellet was washed with 1ml of buffer and re-centrifuged at 18000*g* for 15min. Radioactivity in the wash and the pellets was determined. The total soluble radioactivity and that incorporated into sedimentable material were then calculated.

Metabolism and incorporation of [^{14}C]glucose. Two cultures of *Spirodela* were grown in the dark on medium containing 0.1% glucose and transferred as described above to flasks containing 50μCi of [^{14}C]-glucose (sp. radioactivity 300mCi/mmol) in 10ml of medium with 0.1% glucose. After a further 12h in darkness the *Spirodela* plants were removed, rinsed, dried and samples (approx. 300mg) were weighed. These samples were extracted with 3ml of MCW solution (methanol–chloroform–water, 12:5:3, by vol.) at −20°C for 24h (Cook & Bieleski, 1969). The MCW solution was removed and separated into two phases by the addition of 0.75ml of chloroform and 1ml of water followed by centrifugation. Meanwhile the plant material was extracted with 2ml of MCF solution (methanol–chloroform–water–formic acid, 12:5:2:1, by vol.) at −20°C (Cook & Bieleski, 1969). After 4h the material was homogenized in the MCF solution. The homogenizer was rinsed with 0.5ml of chloroform and then 0.7ml of water and the rinsings were added to the homogenate. After being shaken

and centrifuged at 2000*g* for 15min, two phases were obtained.

The phases from each extraction were separated and the aqueous phases from both parts of each extraction were combined and evaporated to dryness. The dried aqueous extract was redissolved in 500μl of water and the radioactivity in 10-μl samples was determined. The volume of each chloroform phase was measured and the radioactivity in a 50-μl sample was determined.

A portion of each aqueous extract was diluted 100-fold with water and 100μl was applied to a cellulose thin-layer plate (250μm thick) for electrophoresis (Bieleski & Turner, 1966) followed by chromatography in the second dimension in butan-1-ol–water–propionic acid (10:7:5, by vol.). Regions of radioactivity were located by radioautography and eluted, and the associated radioactivity was determined. Known marker compounds were subjected to co-electrophoresis to identify the radioactive materials.

Starch was contained in the interface layer of the MCF extractions. Radioactivity incorporated in starch was determined as described above.

Results

Gross changes in DNA, RNA, soluble protein and starch contents

In a preliminary experiment the gross changes in frond number, frond weight and the DNA, RNA and soluble protein per frond were determined over 4

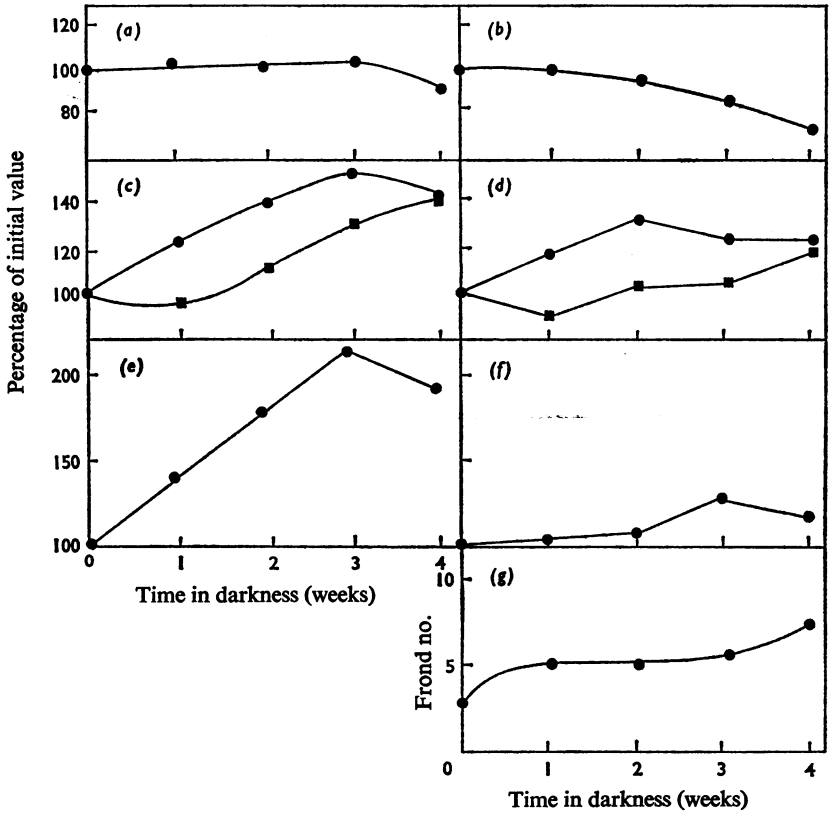

Fig. 1. *Changes in macromolecule content of non-growing Spirodela in darkness*

Four *Spirodela* cultures on basal medium were transferred to darkness. At weekly intervals commencing immediately, one flask was removed and samples of the *Spirodela* were analysed for content of soluble protein, RNA and DNA. The fresh and dry weights/frond were also determined. The changes relative to the initial value of each parameter were then calculated. (*a*) Soluble protein/frond; (*b*) soluble protein/g fresh wt; (*c*) ●, DNA/frond; ■, RNA/frond; (*d*) ●, DNA/g fresh wt.; ■, RNA/g fresh wt.; (*e*) dry weight/frond; (*f*) fresh weight/frond; (*g*) growth of a three-frond plantlet in darkness on basal medium for comparison.

weeks after transfer of light-grown cultures of *Spirodela* into darkness on basal medium without kinetin. The results are presented as percentages of the initial value in Fig. 1(*a–f*).

There was an increase in the DNA and RNA content/frond (Fig. 1*c* and 1*d*) over 4 weeks and an apparent 20% decrease in 'soluble' protein/g fresh weight (Fig. 1*b*) over 3 weeks before growth resumed spontaneously (Fig. 1*g*). However, the decrease in soluble protein was compensated by a comparable increase in the fresh weight/frond (Fig. 1*f*) so that the amount of soluble protein/frond actually remained constant during the dormant period (Fig. 1*a*), then decreased when growth resumed. The large increase in dry weight (Fig. 1*e*) was probably mostly due to starch, which accumulates during the period of dormancy and begins to fall when growth resumes.

Effects of kinetin on protein synthesis

Uptake and incorporation of [^{35}S]sulphate into protein were followed for 72h after *Spirodela* plants were placed in darkness with or without kinetin on low-sulphate medium. The results are presented in Fig. 2. Removal of [^{35}S]sulphate from the medium slowed after 24h, corresponding to a decreased rate of uptake by the plants (Fig. 2*a* and 2*b*). Kinetin did not affect uptake of ^{35}S (Fig. 2*b*), but did increase its incorporation into both soluble and insoluble (i.e. pellet) protein (Fig. 2*c* and 2*d*). The rates of incor-

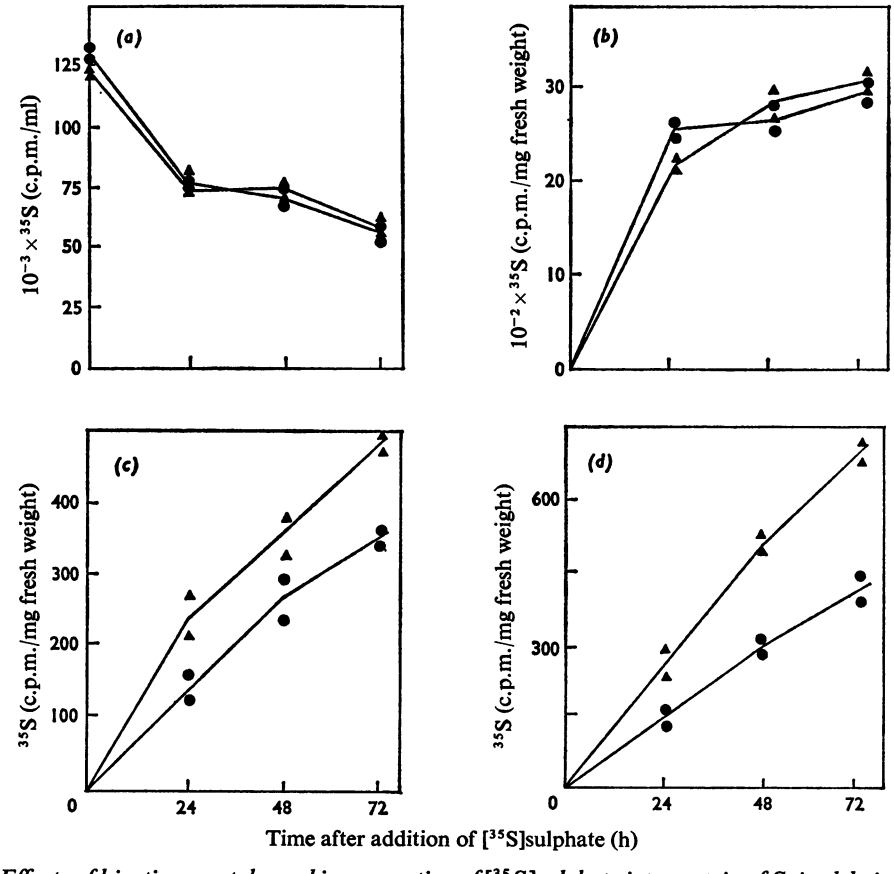

Fig. 2. *Effects of kinetin on uptake and incorporation of* [^{35}S]*sulphate into protein of Spirodela in darkness*

[^{35}S]Sulphate was added to each of two flasks containing *Spirodela* in darkness. One flask contained kinetin (250ng/ml). The disappearance of radioisotope from the medium, the uptake by *Spirodela* and the incorporation into soluble and pellet protein were followed. (For details see the Experimental section.) (*a*) Disappearance of [^{35}S]sulphate from medium; (*b*) uptake of [^{35}S]sulphate by *Spirodela*; (*c*) incorporation of ^{35}S radioactivity into pellet protein; (*d*) incorporation of ^{35}S radioactivity into soluble protein. ▲, Flask with kinetin; ●, control flask.

poration into protein were roughly linear over 3 days (Fig. 2*c* and 2*d*). Incorporation into soluble protein in the non-growing culture was 58% of that in the culture with kinetin (Fig. 2*d*), whereas radioactivity in pellet protein was 68% of that with kinetin (Fig. 2*c*).

Specific radioactivity of sulphate pools

The conclusion that protein synthesis was stimulated in the presence of kinetin in the above experiment was based on the assumption that the specific radioactivity of the sulphate pool did not change on treatment with kinetin. The free sulphate was therefore determined in extracts of *Spirodela* grown with and without kinetin on [^{35}S]sulphate-containing medium in darkness. The specific radioactivity of the free sulphate was then determined and the incorporation of radioactive sulphur into protein was corrected to allow for any changes in specific radioactivity. Plants grown on kinetin contained more total sulphate before the addition of [^{35}S]sulphate, and 24h after [^{35}S]-sulphate addition contained 1.4 times that in the minus-kinetin control. After correction for the lower specific radioactivity of sulphate in plants with kinetin incorporation of sulphur into soluble protein was 2.3 times that in dormant cultures, whereas incorporation into pellet protein was twice that in the absence of kinetin.

Taken together, the above results suggested that considerable protein turnover occurred in dormant plants, for the following reasons. (1) Although synthesis of both soluble and insoluble protein in dormant plants continued at about 50% of the rate in growing plants (Figs. 2*c* and 2*d*) there was no increase in soluble protein over 3 weeks in darkness (Fig. 1*a*). (2) The insoluble protein/frond would have had to increase about 5-fold to account for all of the protein synthesized over 3 weeks if it was synthesized at half the rate of that in growing cultures. However, the dry weight of dormant plants only doubled over 3 weeks, and a large part of this increase was due to starch (see below). Further, increased membrane-bound ribosomes could not account for a 5-fold increase in insoluble protein since the amount of RNA per frond only increased by 60% over 3 weeks.

Effects of various inhibitors on protein synthesis

To confirm that protein synthesis occurs *de novo* in dormant *Spirodela*, cultures were treated with [^{35}S]-sulphate in the presence of antibiotics known to inhibit protein biosynthesis. The minimum concentrations of the antibiotics required to inhibit growth completely in light were predetermined and used in these experiments. Radioactivity incorporated into soluble and insoluble proteins is shown in Table 1 as percentage of total [^{35}S]sulphate taken up by the cultures. Both chloramphenicol and actidione appreciably inhibited protein synthesis. Together the antibiotics decreased the incorporation of [^{35}S]-sulphate into protein by 88% over 24h, supporting the observation that considerable protein synthesis occurred in dormant *Spirodela*.

Time required for stimulation of protein synthesis by kinetin

The time that elapsed between the addition of kinetin to dormant *Spirodela* and the subsequent increase in the rate of protein synthesis was determined. At 1 h after the addition of kinetin to dormant cultures their rate of protein synthesis abruptly doubled and thereafter was similar to that of cultures that had been growing continuously on medium containing kinetin (Fig. 3*b*). In these experiments the uptake of [^{35}S]sulphate had virtually ceased at the time of addition of kinetin (Fig. 3*a*), so that the increased rate of incorporation into protein did not

Table 1. *Effect of inhibitors of protein synthesis in non-growing Spirodela*

For experimental details see the text. Results for cultures growing in darkness with kinetin (250ng/ml) are included for comparison. [^{35}S]Sulphate incorporation is expressed as % of the total ^{35}S radioactivity taken up by each culture. Relative rates of incorporation are the % of the total ^{35}S radioactivity taken up which was incorporated into protein, expressed as % of that incorporated by the minus-kinetin control.

Inhibitor	[^{35}S]Sulphate incorporated (%)		Relative rates of incorporation
	'Soluble' protein	'Pellet' protein	
Kinetin (250ng/ml)	32.9	26.5	148
Control	20.2	20.0	100
Chloramphenicol (20 μg/ml)	12.4	12.7	62
Actidione (1 μg/ml)	2.4	3.9	16
Chloramphenicol (20 μg/ml) and actidione (1 μg/ml)	2.4	2.5	12

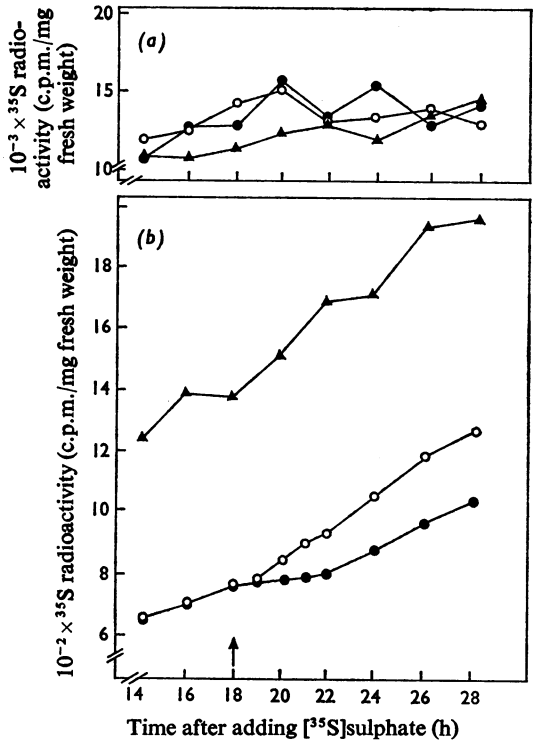

Fig. 3. *Estimation of the time required for stimulation of protein synthesis by kinetin*

Three cultures of *Spirodela* in darkness for 4 days were treated with [^{35}S]sulphate. One flask contained kinetin (250ng/ml). At intervals starting 14h later, samples of *Spirodela* were withdrawn from each flask and radioactivity associated with soluble protein was determined. At 18h after addition of ^{35}S, kinetin (250ng/ml) was added to one flask without kinetin and the sampling continued. (For details see the Experimental section.) (*a*) Uptake of [^{35}S]sulphate by *Spirodela*; (*b*) incorporation of ^{35}S radioactivity into soluble protein. ▲, Cultures on medium with kinetin; ○, cultures on medium with kinetin added 18h after [^{35}S]sulphate; ●, cultures on medium without kinetin. The arrow indicates the time of kinetin addition.

result from a stimulation of the rate of radioisotope uptake. Further, enlargement of the sulphate pool induced by kinetin would have tended to decrease the specific radioactivity of the sulphur entering protein and could not have accounted for any increase in incorporation of [^{35}S]sulphur into protein.

RNA in Spirodela

Spirodela contained approx. 1.6mg of RNA/g fresh wt. of tissue when assayed by the method of Matthews (1958). The percentage base composition of tRNA was C, 27.3; A, 19.8; G, 33.2; U, 19.7, and of rRNA, C, 24.0; A, 23.1; G, 34.1; U, 18.8. The two RNA species were isolated by chromatography on methylated bovine serum albumin columns and base compositions were determined by electrophoresis after alkaline hydrolysis (Smith, 1955).

Changes in RNA synthesis with kinetin

The rate of uptake and incorporation of [^{3}H]-uridine into RNA in growing and non-growing *Spirodela* in darkness is shown in Fig. 4. Kinetin did not affect the uptake of uridine by *Spirodela* (Fig. 4*a*) whereas the rate of incorporation into RNA in the culture with kinetin was 3-fold greater than that in non-growing plants (Fig. 4*b*).

When the radioactive RNA was fractionated by chromatography on methylated bovine serum albumin columns it was found that kinetin stimulated [^{3}H]uridine incorporation into tRNA and rRNA to nearly equal extents and that the specific radioactivity of rRNA was slightly greater than that of tRNA. The values (c.p.m./E_{260}) observed in a typical experiment were: minus kinetin, tRNA 462, rRNA 448; plus kinetin, tRNA 1320, rRNA 1520.

If *Spirodela* growing in darkness with and without kinetin were treated with [^{32}P]P_i for 4h and the RNA was extracted and fractionated on methylated bovine serum albumin columns, synthesis of 18S rRNA appeared to be slightly greater than that of tRNA and 28S rRNA in the presence of kinetin.

Time required for stimulation of RNA synthesis by kinetin

After the addition of kinetin to dormant *Spirodela* the rate of RNA synthesis abruptly increased 3-fold to equal that of cultures grown continuously on kinetin (Fig. 5*b*). The increased rate was not attributable to increased rate of uptake of [^{3}H]uridine, since the latter was low and steady in cultures with or without kinetin, throughout the experiment (Fig. 5*a*). Attempts to determine the exact time-lag before the rate of RNA synthesis increased showed that the increase occurred 30–60min after the addition of kinetin. A more precise estimate of the lag could not be obtained. It seems unlikely that an abrupt change could have occurred in the uridine pool of sufficient magnitude to explain the observed increase in RNA synthesis.

Turnover of RNA in growing and non-growing Spirodela

The rate-constant of degradation of RNA in cultures of *Spirodela* was found by determining the difference between the rate constant of gross synthesis and the rate constant of net accumulation of RNA (Trewavas, 1970).

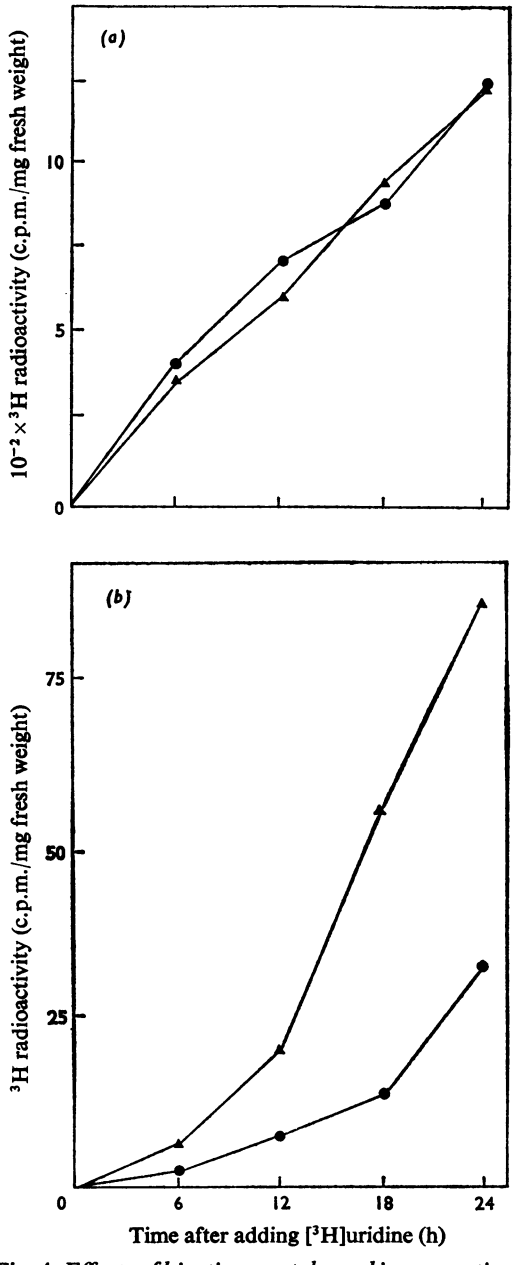

Fig. 4. *Effects of kinetin on uptake and incorporation of [^3H]uridine into RNA of Spirodela in darkness*

[^{3}H]Uridine was added to each of two flasks containing *Spirodela* in darkness. One flask contained kinetin (250ng/ml). The uptake of radioactivity by the *Spirodela* and incorporation into RNA were followed. (*a*) Uptake of [^{3}H]uridine; (*b*) incorporation of [^{3}H]uridine into RNA. ▲, Radioactivity with kinetin; ●, radioactivity in control. For details see the Experimental section.

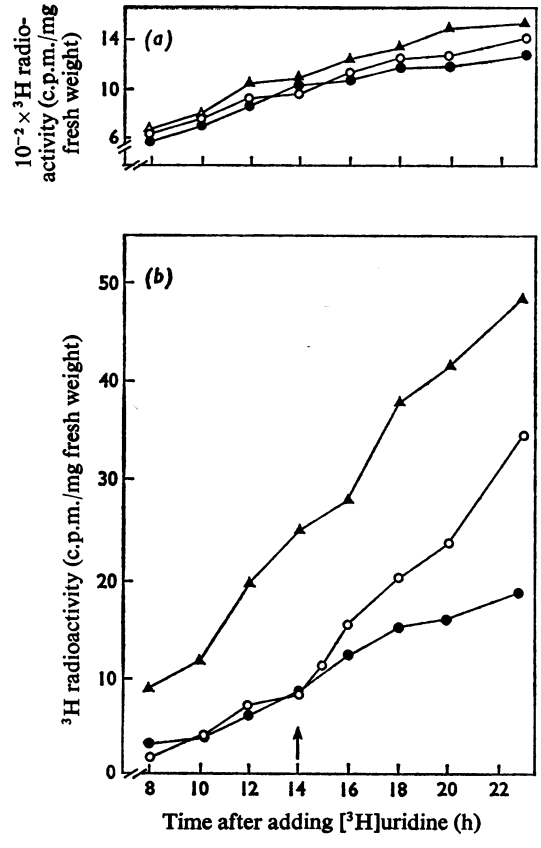

Fig. 5. *Estimation of time required for stimulation of RNA synthesis by kinetin*

The protocol for this experiment was essentially the same as that for Fig. 3, but with [^{3}H]uridine as radioactive precursor. Kinetin was present in one flask throughout and was added to a second flask 14h after addition of [^{3}H]uridine to each flask. For details see the Experimental section. (*a*) Uptake of [^{3}H]uridine by *Spirodela*; (*b*) incorporation of [^{3}H]uridine into RNA. ▲, Cultures on medium with kinetin: o, cultures on medium with kinetin added 14h after [^{3}H]-uridine; ●, cultures on medium without kinetin. The arrow indicates the time of kinetin addition.

Measurement of gross RNA synthesis. The rate of gross RNA synthesis was determined by following the rate of dilution of radioactive RNA by non-radioactive precursor, after a 'pulse' of radioactive precursor (see the Experimental section). This procedure avoids complications arising from variations in precursor pools (see Trewavas, 1970). The rate constant for gross synthesis of RNA was obtained by expressing in natural logarithms the slope of the semi-logarithmic plot of the (decreasing) specific

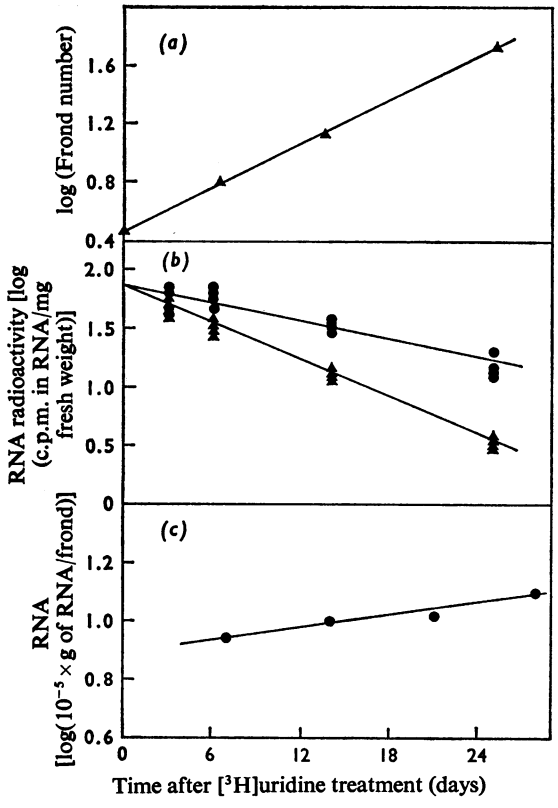

Fig. 6. *Determination of rates of gross and net synthesis of RNA by growing and non-growing Spirodela*

Full details are given in the text. (*a*) Increase in frond number (i.e. RNA)/culture in growing *Spirodela* in darkness. (*b*) Decrease in RNA-associated ^3H radioactivity/mg fresh wt. in growing and non-growing *Spirodela* in darkness after 4 days uptake of [^3H]-uridine followed by excess of non-radioactive uridine. (*c*) Increase in RNA/frond in non-growing *Spirodela* in darkness. ▲, Kinetin culture; ●, control culture.

radioactivity of radioactive RNA versus time, after the addition of non-radioactive RNA precursor (see Reiner, 1953). As described in the Experimental section, the ^3H radioactivity/mg fresh weight of *Spirodela* was determined for growing (plus kinetin) and non-growing (minus kinetin) cultures over 25 days in darkness after 4 days uptake of [^3H]uridine and transfer to excess of non-radioactive uridine (Fig. 6*b*). Since the amount of RNA/mg fresh wt. in the growing culture remained constant the slope of the plot (Fig. 6*b*) is the same as that of specific radioactivity of the RNA versus time. In Fig. 6(*b*), showing ^3H radioactivity in RNA/mg fresh wt. versus time for the non-growing culture, the values are

Table 2. *Rate constants for synthesis, net increase and degradation of RNA in growing and non-growing Spirodela*

Rate constants for synthesis and degradation of RNA in growing (+kinetin) and non-growing (control) cultures in darkness were calculated as described in the text.

Culture	Gross synthesis	Net increase	Degradation
+Kinetin	0.13	0.11	0.02
Control	0.06	0.01	0.05

corrected to allow for the slow accumulation of RNA/mg fresh wt. in the culture (Fig. 1*d*). The rate of decrease of specific radioactivity of the RNA was then determined (Table 2).

Net accumulation of RNA. Since the net accumulation of RNA in growing cultures of *Spirodela* is compensated for by growth, the rate constant for net accumulation of RNA may be obtained from the slope of the semi-logarithmic plot of frond number/culture versus time. The slope of this line (Fig. 6*a*), expressed as a natural logarithm, is the rate constant for net accumulation of RNA (Table 2) (see Trewavas, 1970). In non-growing *Spirodela*, RNA/frond increases slowly (Fig. 1*c*). Since there is no increase in frond number, the rate constant for net accumulation of RNA may be obtained from the semi-logarithmic plot of mg of RNA/frond versus time. The slope of this line (Fig. 6*c*), expressed as a natural logarithm, is the rate constant for the net accumulation of RNA in non-growing cultures (Table 2).

The difference between the rate constants of gross and net synthesis of RNA for each culture (Table 2) is the rate constant for degradation of RNA in those plants. Table 2 shows that kinetin increased RNA synthesis by over 100% and decreased the rate of degradation of RNA by 60%.

Free and membrane-bound ribosomes

The ratio of free to membrane-bound ribosomes in dormant and growing *Spirodela* in darkness was determined by using plants pre-labelled with [^{32}P]P_i for 7 days. The plants were homogenized and the homogenates centrifuged at 18000*g* and 180000*g*. The radioactivity in RNA extracted from the individual pellets was determined. Table 3 shows that in cultures growing with kinetin 50% of the ribosomes were attached to membranes, whereas in non-growing cultures 66% were so attached. These ribosomes sedimented at 18000*g*. Altering the K^+ ion concentration between 1 and 100 mM did not affect the proportions of bound ribosomes. Thin-section electron micrographs, cut from glutaraldehyde-fixed Epon-embedded material, were also examined. The 18000*g*

Table 3. *Distribution of free and membrane-bound ribosomes in growing and non-growing Spirodela*

For experimental details see the text. Bound radioactivity is that in RNA associated with 18000*g* pellets, and free radioactivity is that in RNA associated with 180000*g* pellets.

	$10^{-5} \times {}^{32}P$ radioactivity (c.p.m./g fresh wt.)		Percentage distribution of ribosomes	
Culture	Bound	Free	Bound	Free
Minus kinetin	4.70	2.73	63	37
Plus kinetin	4.08	4.46	48	52

pellets contained many membranes with ribosomes attached as well as cell-wall material and plastid fragments. The 180000*g* pellet consisted of free ribosomes together with small spheres of smooth membrane. Payne & Boulter (1969*a*,*b*) have shown that cells undergoing rapid division in *Vicia* cotyledons have mostly free ribosomes, whereas in non-dividing differentiating cells more ribosomes are membrane-bound.

Time required for stimulation of DNA synthesis by kinetin

The rates of [^{3}H]thymidine incorporation into DNA increased abruptly 1h after addition of kinetin to one of two flasks of dormant *Spirodela* (Fig. 7*b*). Until kinetin was added the rates were identical in both flasks and uptake of thymidine had virtually ceased (Fig. 7*a*). The amount of total soluble radioactivity decreased slowly in the minus-kinetin control culture during the period of kinetin treatment, as did the rate of incorporation into DNA.

After uptake of [^{14}C]thymidine plants were mounted on a card and radioautographed. Examination of the radioautographs showed that radioactivity was present over the whole area of fronds, but much more was concentrated at the bases of expanding fronds and in incipient fronds. In these meristematic regions cells are smaller and more DNA is present/unit area than in the frond lamellae. Plants treated with kinetin during exposure to radioactive thymidine showed a greater concentration of radioactivity in the meristematic regions, especially in primordia of fronds not yet visible beyond the parent frond.

Effect of kinetin on starch in growing and non-growing Spirodela

In the presence of kinetin, the amount of starch/g fresh wt. of *Spirodela* decreased by 50% during the first 2 days after transfer of plants to darkness and

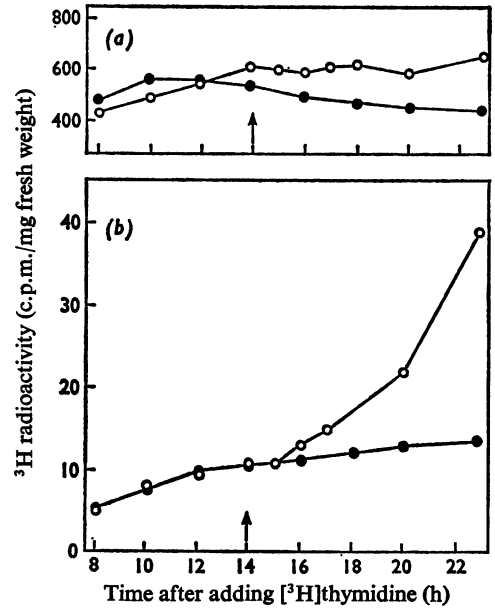

Fig. 7. *Stimulation of DNA synthesis by kinetin addition to non-growing Spirodela*

Two *Spirodela* cultures were placed in darkness. After 4 days [^{3}H]thymidine was added to each culture and samples were removed at intervals after a further 8h. ^{3}H radioactivity in DNA was determined in these samples. At 14h after the addition of radioisotope, kinetin was added to one culture and the sampling time-course continued. (*a*) Uptake of [^{3}H]thymidine, (total radioactivity/mg fresh wt); (*b*) incorporation of [^{3}H]thymidine into DNA. ●, Control culture, ○, culture with kinetin added 14h after [^{3}H]thymidine. The arrow indicates the time of kinetin addition.

then increased slightly over the next 4 days (Fig. 8*a*). In cultures transferred to darkness in the absence of kinetin, the amount of starch decreased initially but began to increase rapidly at about the time growth ceased (Fig. 8*a*). After 6 days in darkness the non-growing plants contained three times as much starch as photosynthesizing plants on the same medium. It appeared that the increase in the amount of starch was due to an inability of non-growing plants to utilize the glucose entering from the medium. This was confirmed when plants were transferred to darkness on medium containing kinetin and 1 μM-5-fluorodeoxyuridine to stop growth. In the presence of fluorodeoxyuridine starch accumulated after a 1-day lag, despite the presence of kinetin (Fig. 8*b*).

Starch accumulated on basal medium in darkness without kinetin was still present when plants were transferred to medium lacking glucose in darkness

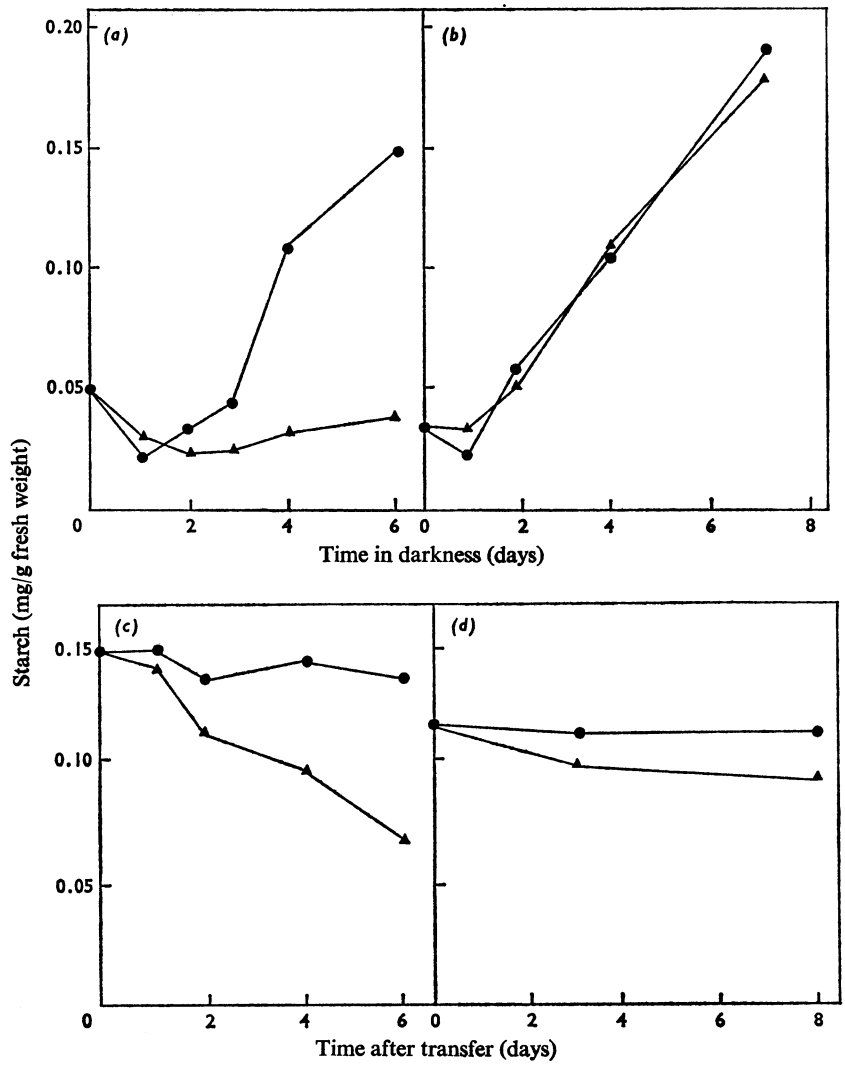

Fig. 8. *Accumulation and mobilization of starch by Spirodela in darkness*

For experimental details see the text. (*a*) and (*b*) Equal quantities of light-grown *Spirodela* were transferred to each of two pairs of flasks containing medium with 1% glucose. One flask of each pair contained kinetin (250ng/ml). One pair of flasks also contained 1 μM-5-fluorodeoxyuridine. The flasks were placed into darkness and samples of *Spirodela* (approx. 200mg) were withdrawn under dim-green light at intervals. The starch content of the *Spirodela* in each sample was determined. (*a*) ●, Minus kinetin; ▲, plus kinetin. (*b*) ●, Minus kinetin, plus fluorodeoxyuridine; ▲, plus kinetin, plus fluorodeoxyuridine. (*c*) and (*d*) A culture of *Spirodela* was left in darkness for 6 days on basal medium containing 1% glucose. Equal quantities of the *Spirodela* were then transferred under dim-green light to two pairs of flasks containing medium without glucose. One flask of each pair contained kinetin (250ng/ml). One pair of flasks also contained 1 μM-5-fluorodeoxyuridine. The flasks were replaced in darkness and samples of *Spirodela* (approx. 200mg) were withdrawn for starch determination at intervals. (*c*) ●, Minus kinetin; ▲, plus kinetin. (*d*) ●, Minus kinetin, plus fluorodeoxyuridine; ▲, plus kinetin, plus fluorodeoxyuridine.

for 6 days. When kinetin was present, the starch content decreased to less than half over the 6 days (Fig. 8*c*). However, this decrease in starch content reflected the growth of the culture more than the net loss of starch, since new fronds formed in the presence of kinetin, despite the lack of glucose. When these

experiments were repeated with fluorodeoxyuridine (10 μM) in each flask lacking glucose, treatment with kinetin still decreased the starch content by 20% (per g fresh wt.) although no new fronds appeared in the cultures. Again little loss of starch occurred in the absence of kinetin (Fig. 8*d*).

Effects of kinetin on uptake, incorporation and distribution of [^{14}C]glucose

Kinetin did not affect the uptake of [^{14}C]glucose by *Spirodela* in darkness. When the distribution of ^{14}C radioactivity was examined after fractionation of extracts of *Spirodela* grown on [^{14}C]glucose (Cook & Bieleski, 1969) more [^{14}C]glucose was converted into starch in non-growing plants than in growing plants. The growing cultures incorporated more radioactivity into organic-phase-soluble material (Table 4), which includes lipids and lipid phosphates (Cook & Bieleski, 1969). The radioactive compounds in the aqueous phase of each extract were further fractionated by t.l.c., located by radioautography, and the compounds were identified by co-chromatography with known standards. Table 5 shows the distribution of ^{14}C radioactivity in each extract. There was a substantial increase in ^{14}C radioactivity in sucrose and polysaccharide and a decrease of ^{14}C in fructose in the presence of kinetin.

Table 4. *Distribution of radioactivity in extracts of Spirodela grown on [^{14}C]glucose*

For experimental details see the text. Results are % of the total radioactivity associated with the three phases. Kinetin did not affect the uptake of [^{14}C]-glucose by the *Spirodela* and there was a total of 45000c.p.m. of ^{14}C/mg fresh wt. in the three phases from each extraction.

Phase	Control	Kinetin
Aqueous	91.6	92.2
Organic	3.8	6.4
Starch	4.6	1.4

Discussion

Growth of *Spirodela* ceases after transfer of plants from light to dark in the absence of kinetin. The plants then remain apparently dormant for 3–4 weeks before spontaneously resuming growth in darkness. Despite their failure to grow, synthesis of DNA, RNA and protein continued in dormant plants at rates about 50% below those in kinetin-treated plants.

There appeared to be considerable turnover of DNA and RNA in the dormant plants, because the total amounts of these materials stayed relatively constant in spite of their continued synthesis. The

Table 5. *Distribution of ^{14}C radioactivity among compounds in the aqueous phase of extracts of Spirodela grown on [^{14}C]glucose*

For experimental details see the text. The ratio is corrected to equal c.p.m. applied to chromatograms.

Compound	10^{-2} × Associated radioactivity (c.p.m.) Control	+Kinetin	Ratio (kinetin/control)
Sugar phosphates	387	470	1.17
Polysaccharide	123	142	1.11
Polysaccharide + maltose	119	294	2.37
Sucrose	194	591	2.93
Glucose	25.4	33.8	1.28
Fructose	35.1	18.9	0.52
Citrate	49.1	57.4	1.12
Malate	142	121	0.82
Glycollate + succinate	231	163	0.68
Asparagine	124	16.5	0.13
Aspartate	123	109	0.85
Glutamine	12.4	10.8	0.84
Serine	14.4	17.6	1.17
Glutamate	121	135	1.07
Alanine	71.3	61.2	0.82
Arginine	45.9	34.9	0.73
γ-Aminobutyrate	84.2	64.9	0.74
Recovery (%)	65	70	

results also supported the conclusion that considerable protein turnover occurred in the dormant plants. Starch accumulated in non-growing *Spirodela*, apparently as a consequence of growth inhibition, since it also accumulated in plants whose growth was stopped by fluorodeoxyuridine. Kinetin failed to affect growth or starch accumulation in the presence of fluorodeoxyuridine. Reid (1968) also found that inhibiting *Spirodela* growth by calcium starvation caused starch to accumulate, and Tasseron-de Jong & Veldstra (1971) found that starch accumulated in *Lemna minor* when growth was inhibited by an excess of 6-benzyladenine.

Addition of kinetin to dormant cultures of *Spirodela* in darkness increased DNA, RNA and protein synthesis after 1 h, and initiated growth. Synthesis of both tRNA and rRNA increased. A decrease in RNA degradation was also demonstrable, and less ribosomes were associated with membranes in the presence of kinetin in darkness. A stimulation of RNA synthesis in *L. minor* on water by 6-benzyladenine in light was found by Trewavas (1970). However, under his conditions RNA degradation was also increased.

By using the above procedures we could not demonstrate a specific effect of kinetin on DNA, RNA or protein synthesis, or on starch synthesis. There was also no evidence of a major change or a block in intermediary metabolism in plants without kinetin. Increased ^{14}C radioactivity present in asparagine in non-growing plants (Table 5) was probably not a direct consequence of kinetin action, since increased asparagine is also observed in *Spirodela* growing on excess of N (E. G. Bollard, personal communication). The results suggest the general conclusion that intermediary metabolism operates at similar velocities in dormant and growing *Spirodela* despite the different rates of synthesis of macromolecules. We could not exclude an early effect of kinetin on the transcription or translation of a specific mRNA which is subject to control by kinetin. However, it appeared that DNA, RNA and protein synthesis increased simultaneously 1 h after addition of kinetin to dormant plants as a consequence of restored growth potential. The amount of [^{14}C]kinetin reaches a plateau in *Spirodela* 30–60 min after its addition to cultures (McCombs & Ralph, 1972).

There are considerable similarities between the responses of *Spirodela* after transfer to darkness, the effects of stringent control after 'shift-down' culture of bacteria (Maaløe & Kjeldgaard, 1966) and pleiotypic control after 'step-down' of animal tissues (Hershko *et al.*, 1971). Stringent and pleiotypic responses to 'step-down' culture changes include decreased rates of growth, DNA, RNA and protein synthesis and increased protein degradation. These effects also occur when *Spirodela* is transferred to darkness. The reversal of these processes after addition of kinetin to *Spirodela* in darkness resembles the reversal of stringent and pleiotypic control by 'shift-up' conditions (see Maaløe & Kjeldgaard, 1966; Edlin & Broda, 1968; Hershko *et al.*, 1971).

There have been numerous suggestions about the mechanism of control in the stringent and pleiotypic responses. The co-ordination of the various processes affected has led to the idea that they are controlled together by a common mediator or process, perhaps even by the same process or mediator in bacteria and animal cells (Hershko *et al.*, 1971). Factors suspected of controlling the stringent response have been reviewed by Edlin & Broda (1968). Cashel & Kalbacher (1970) have implicated the unusual nucleotide ppGpp in stringent control in bacteria. However, its role is still somewhat controversial and it was not detected in 'step-down' HeLa cells (Smulson, 1970).

The addition of insulin (or serum) to animal cells in culture in deficient medium can stimulate DNA, RNA and protein synthesis and growth (Tata, 1966; Hershko *et al.*, 1971). Insulin appears to inhibit adenylate cyclase (Hepp *et al.*, 1969; Menahan & Wieland, 1969) and stimulate adenosine 3′:5′-cyclic monophosphate (cyclic AMP) diesterase (Senft *et al.*, 1968), thereby decreasing the concentration of cyclic AMP in animal cells. Low concentrations of cyclic AMP have been shown to be closely related to increased growth potential in animal cells (Sheppard, 1971, 1972). In contrast, in bacteria cyclic AMP stimulates mRNA synthesis (Zubay *et al.*, 1970). Therefore if there is a single mediator of the stringent and pleiotypic responses it seems unlikely that this is cyclic AMP. However, cyclic AMP acts by modifying the action of specific protein kinases (cf. Jöst & Rickenberg, 1971; Greengard *et al.*, 1971). We suggest that the control of specific protein kinase(s) action may be the significant factor in both the stringent and pleiotypic response. In other studies (P. J. A. McCombs & R. K. Ralph, unpublished work) we have found that cytokinins modify the activity of protein kinase(s) from leaf and carrot tissue. Thus all three processes, stringent control in bacteria, pleiotypic control in animal cells and growth control in *Spirodela* and other plants, could be aspects of one universal control mechanism, protein phosphorylation mediated by protein kinases which are sensitive to a variety of specific effector molecules.

References

Anderson, J. W. & Rowan, K. S. (1966) *Biochem. J.* **98**, 401
Battacharyya, J. & Roy, S. L. (1969) *Biochem. Biophys. Res. Comm.* **35**, 606
Berridge, M. V. & Ralph, R. K. (1969) *Biochim. Biophys. Acta* **182**, 266
Berridge, M. V., Ralph, R. K. & Letham, D. S. (1970) *Biochem. J.* **119**, 75
Bieleski, R. L. & Turner, N. A. (1966) *Anal. Biochem.* **17**, 278

Burrows, W. J., Skoog, F. & Leonard, N. H. (1971) *Biochemistry* **10**, 2189
Cashel, M. & Kalbacher, B. (1970) *J. Biol. Chem.* **245**, 2309
Cook, A. R. & Bieleski, R. L. (1969) *Anal. Biochem.* **28**, 428
Davies, J. W. & Cocking, E. C. (1967) *Biochem. J.* **104**, 23
Edlin, G. & Broda, P. (1968) *Bacteriol. Rev.* **32**, 206
Filner, P., Wray, J. L. & Varner, J. E. (1969) *Science* **165**, 358
Fox, J. E. & Chen, C. M. (1967) *J. Biol. Chem.* **242**, 4490
Fox, J. E. & Chen, C. M. (1968) in *Biochemistry and Physiology of Plant Growth Substances* (Whitman, F. & Stetterfield, G., eds.), p. 777, Runge Press, Ottawa
Giles, K. W. & Myers, A. (1965) *Nature (London)* **206**, 93
Gorham, P. R. (1950) *Can. J. Bot.* **28**, 356
Greengard, P., Kuo, J. F. & Miyamoto, E. (1971) *Advan. Enzyme Regul.* **9**, 113
Guttman, R. J. (1957) *Biochem. Cytol.* **3**, 129
Helgeson, J. P. (1968) *Science* **161**, 974
Hepp, K. D., Menahan, L. A., Wieland, O. & Williams, R. H. (1969) *Biochim. Biophys. Acta* **184**, 554
Hershko, A., Marmont, P., Shields, R. & Tomkins, G. M. (1971) *Nature New Biol. (London)* **232**, 206
Jensen, W. A., Pollock, E. G., Healey, P. & Ashton, M. (1964) *Exp. Cell Res.* **33**, 523
Johnson, C. M. & Ulrich, A. (1959) *Calif. Agr. Exp. Sta. Bull.* **776**, 25
Jöst, J. P. & Rickenberg, H. V. (1971) *Annu. Rev. Biochem.* **40**, 741
Key, J. L. (1969) *Annu. Rev. Plant Physiol.* **20**, 449
Letham, D. S. (1967*a*) *Planta* **74**, 228
Letham, D. S. (1967*b*) *Annu. Rev. Plant Physiol.* **18**, 349
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265
Maaløe, O. & Kjeldgaard, N. O. (1966) *Control of Macromolecular Synthesis*, p. 284, W. A. Benjamin, New York
McCombs, P. J. A. & Ralph, R. K. (1972) *Planta* in the press
McCready, R. M., Guggolz, J., Silviera, V. & Owens, H. S. (1950) *Anal. Chem.* **22**, 1156
Matthews, R. E. F. (1958) *Virology* **5**, 192
Matthysee, A. G. & Abrams, M. (1970) *Biochim. Biophys. Acta* **199**, 511
Mejbaum, W. (1939) *Hoppe-Seyler's Z. Physiol. Chem.* **258**, 117
Menahan, L. A. & Wieland, O. (1969) *Eur. J. Biochem.* **9**, 55
Mizrahi, Y., Amir, J. & Richmond, A. E. (1970) *New Phytol.* **69**, 355
Mothes, K. (1964) *Colloq. Int. Cent. Nat. Rech. Sci.* **123**, p. 131
Oota, Y. (1964) *Annu. Rev. Plant Physiol.* **15**, 17
Osborne, D. J. (1962) *Plant Physiol.* **37**, 595
Paranjothy, K. P. & Wareing, P. F. (1971) *Planta* **99**, 112
Payne, P. I. & Boulter, D. (1969*a*) *Planta* **84**, 263
Payne, P. I. & Boulter, D. (1969*b*) *Planta* **87**, 63
Reid, M. S. (1968) Ph.D. Thesis, University of Auckland
Reiner, J. M. (1953) *Arch. Biochem. Biophys.* **46**, 53
Report on Hungarian Academy of Sciences Conference (1971) *Nature New Biol. (London)* **233**, 192
Richmond, A. E., Bach, A. & Sachs, B. (1970) *Planta* **90**, 57
Richmond, A. E., Sachs, B. & Osborne, D. J. (1971) *Physiol. Plant.* **24**, 176
Rijven, A. H. G. C. & Parkash, V. (1971) *Plant Physiol.* **47**, 59
Senft, G., Schultz, G., Munske, K. & Hoffman, M. (1968) *Diabetologia* **4**, 322
Sheppard, J. R. (1971) *Proc. Nat. Acad. Sci. U.S.* **68**, 1316
Sheppard, J. R. (1972) *Nature New Biol. (London)* **236**, 141
Smith, J. D. (1955) in *The Nucleic Acids* (Chargaff, E. & Davidson, J. N., eds.), vol. 1, p. 267. Academic Press, New York
Smulson, M. E. (1970) *Biochim. Biophys. Acta* **199**, 537
Sugiura, M., Umemura, K. & Oota, Y. (1962) *Physiol. Plant.* **15**, 457
Tasseron-de Jong, J. G. & Veldstra, H. (1971) *Physiol. Plant.* **24**, 235
Tata, J. R. (1966) *Progr. Nucl. Acid Res. Mol. Biol.* **5**, 191
Trewavas, A. (1968) *Progr. Phytochem.* **1**, 113
Trewavas, A. (1970) *Plant Physiol.* **45**, 742
Zubay, G., Schwartz, D. & Beckwith, J. (1970) *Proc. Nat. Acad. Sci. U.S.* **66**, 104