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THE EFFECT OF KINETIN ON CHLOROPHYLL SYNTHESIS IN AGEING ETIOLATED BARLEY LEAVES EXPOSED TO LIGHT

A. K. STOBART and P. R. SHEWRY
Department of Botany, The University, Bristol BS8 1UG
and
D. R. THOMAS
Department of Botany, The University, Newcastle-upon-Tyne

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Abstract—Etiolated barley seedlings lose the ability to produce chlorophyll and soluble protein on exposure to light with increasing age. Similarly, the production of δ-aminolaevulinic acid-dehydratase and succinyl-CoA synthetase is decreased in older etiolated leaves exposed to light. The rate of protochlorophyllide$_{652}$ regeneration decreased well before the rates of exogenous δ-aminolaevulinic acid conversion to protochlorophyllide$_{632}$ was affected by ageing. Application of kinetin retarded these ageing symptoms in the etiolated leaves.

INTRODUCTION

AGEING dark-grown barley seedlings lose the ability to synthesize chlorophyll to any great extent and show a diminished photosynthetic ability[1] and the level of protein nitrogen in the leaves declines after about 8 days.[2] Moreover, Simon[3] reports that attached cucumber cotyledons in the dark lose chlorophyll and protein and that this loss is greater in detached cotyledons. In the present work, attached etiolated leaves of barley seedlings have been examined for chlorophyll precursors and for their ability to synthesize such precursors from ALA* and the activity *in vitro* of other enzymes associated with chlorophyll synthesis, and break down is also reported. The effects of kinetin treatment on all these symptoms of ageing have been investigated.

RESULTS

Dark-grown etiolated barley seedlings from 4- to 20-day-old were placed under continuous illumination for 16 hr and chlorophyll, protein, ALA-dehydratase and succinyl-CoA synthetase activity determined. Maximum chlorophyll production occurred in leaves of 6-day-old seedlings and thereafter rapidly decreased until in the 20-day-old seedlings little chlorophyll production was detected (Fig. 1a). The increase in protein content, ALA-dehydratase and succinyl-CoA synthetase activities on illumination also decreased with the age of the etiolated seedlings. In leaves sprayed daily with kinetin, the amounts of chlorophyll synthesized on exposure to light still declined with age but not to such an extent as in

* Abbreviations: ALA, δ-aminolaevulinic acid; P_{652}, protochlorophyllide with maximum *in vivo* absorption at 652 nm; P_{684}, chlorophyllide absorbing at 684 nm; P_{672}, chlorophyllide absorbing at 672 nm; P_{632}, pigment absorbing at 632 nm synthesized from exogenous ALA.

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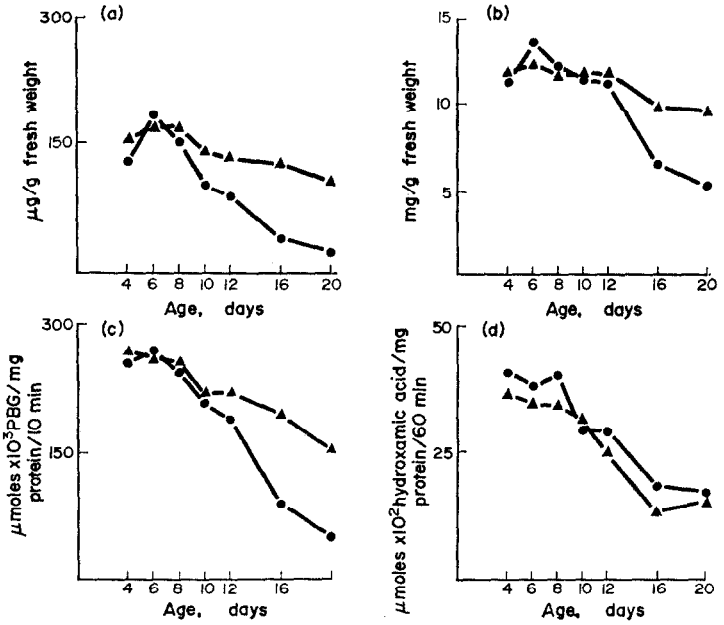

FIG. 1. DARK-GROWN BARLEY SEEDLINGS SPRAYED WITH WATER (●) OR KINETIN (▲) EACH DAY. These were exposed to light for 16 hr and immediately analysed for (a) chlorophyll, (b) protein, (c) ALA-dehydratase activity, and (d) succinyl-CoA synthetase activity. The seedlings were illuminated in this way and analysed for these components at various intervals between 4 and 20 days after initial germination.

the controls (Fig. 1a). Similarly, kinetin treatment delayed the decrease in protein content (Fig. 1b) and ALA-dehydratase activity (Fig. 1c). Little difference was observed between succinyl-CoA synthetase activity in leaves treated with kinetin and the controls (Fig. 1d).

The levels of endogenous protochlorophyll and protochlorophyllide in leaves of etiolated seedlings treated with either kinetin or water are given in Fig. 2. Kinetin treatment

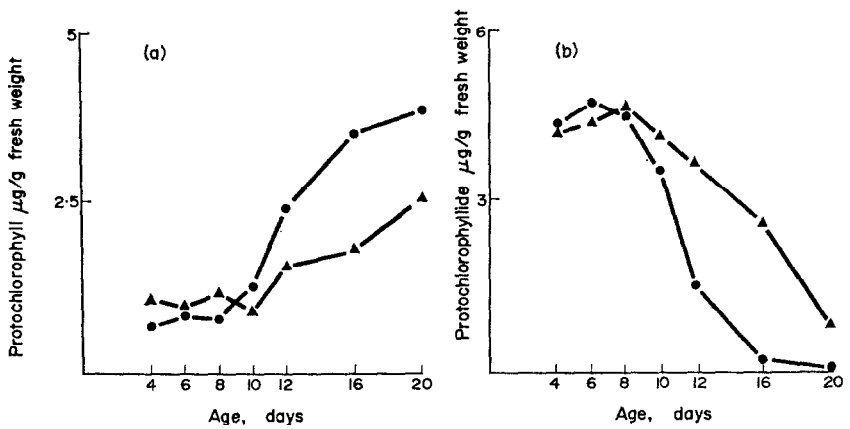

FIG. 2. THE EFFECT OF KINETIN ON (a) PROTOCHLOROPHYLL AND (b) PROTOCHLOROPHYLLIDE LEVELS IN AGEING DARK-GROWN BARLEY SEEDLINGS.
●—Water controls; ▲—kinetin treated.

depresses the level of protochlorophyll while preserving to some extent the levels of protochlorophyllide present in the leaves. The native protochlorophyllide$_{652}$ was converted *in vivo* to a P_{684} absorbing form (chlorophyllide) which within 8 min changed to a P_{672} absorbing form (Fig. 3a). Kinetin and water-treated leaves of various ages were given a brief

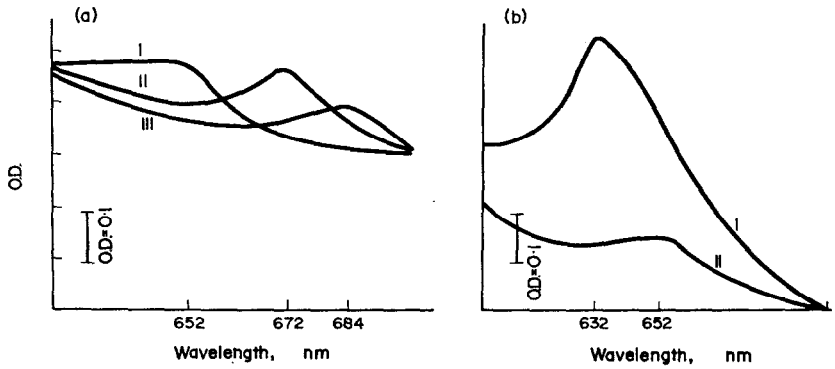

FIG. 3. *In vivo* ABSORPTION SPECTRA OF 7-DAY OLD DARK GROWN BARLEY SEEDLINGS. (a) (i) Etiolated leaves; (ii) etiolated leaves after 60 sec light treatment; (iii) etiolated leaves 8 min after light treatment. (b) (i) Etiolated leaves; (ii) absorption spectrum 2 hr after the addition of 0·01 M ALA solution to the cuvette (see Experimental).

light treatment to convert all the P_{652} to the P_{684} and P_{672} form. The rates of P_{652} regeneration were then measured on return to the dark. The capacity to regenerate P_{652} rapidly diminished in etiolated leaves older than 6 days (Fig. 4a). The lag phase for P_{652} regeneration

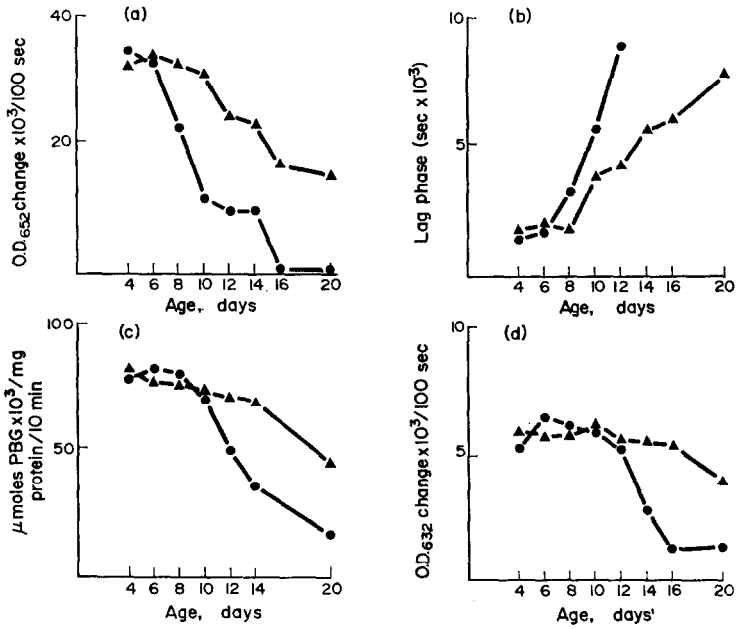

FIG. 4. THE EFFECT OF KINETIN ON (a) RATES OF PROTOCHLOROPHYLLIDE (P_{652}) REGENERATION; (b) THE LAG PHASE BEFORE REGENERATION; (c) ALA–DEHYDRATASE ACTIVITY; AND (d) P_{632} PRODUCTION FROM EXOGENOUS ALA, IN AGEING DARK-GROWN BARLEY SEEDLINGS.
●—Water controls; ▲—kinetin treated.

increased rapidly after 6 days (Fig. 4b). Kinetin treatment delayed both the reduction in rate of regeneration and the lag phase. The loss in synthetic ability was further investigated by following the production *in vivo* of protochlorophyllide$_{632}$ (P_{632}) synthesized from exogenous ALA. Etiolated leaves in a glass cuvette containing ALA (see Experimental) showed an increase in absorbance at 632 nm (Fig. 3b). The rates of P_{632} synthesis from supplied ALA in ageing barley seedlings are given in Fig. 4d. The rate of synthesis was decreased in 10- to 12-day-old etiolated seedlings. Kinetin-treated seedlings retained the ability to synthesize chlorophyll precursors from exogenous ALA. ALA-dehydratase activity declined after 8–10 days etiolation (Fig. 4c) and followed closely the loss in ability to convert ALA to protochlorophyllide$_{632}$. Kinetin treatment retarded the loss in ALA-dehydratase activity in ageing etiolated seedlings (Fig. 4c).

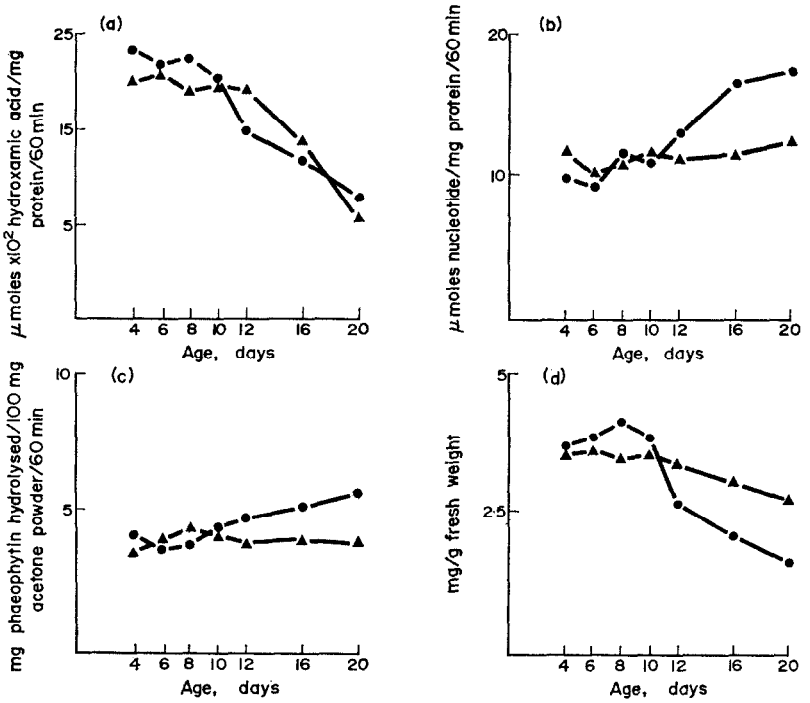

FIG. 5. THE EFFECT OF KINETIN ON (a) SUCCINYL-Co A SYNTHETASE; (b) RNASE (c) CHLOROPHYLLASE; AND (d) SOLUBLE PROTEIN IN AGEING DARK-GROWN BARLEY SEEDLINGS.
●—Water controls; ▲—kinetin treated.

Protein levels (Fig. 5d) and succinyl-CoA synthetase activity (Fig. 5a) in the dark-grown seedlings decreased after 8 days. Kinetin treatment conserved, to some degree, the soluble protein level but had no detectable effect on succinyl-CoA synthetase activity. In ageing etiolated seedlings, RNAse (Fig. 5b) and chlorophyllase activity (Fig. 5c) increased, the increases being diminished by kinetin treatment.

Sulpholipid synthesis in the dark-grown seedlings was determined by the incorporation of $^{35}SO_4^{2-}$. The maximum incorporation was found in 4- to 8-day-old leaves. Kinetin treatment extended the period of sulpholipid synthesis (Fig. 6).

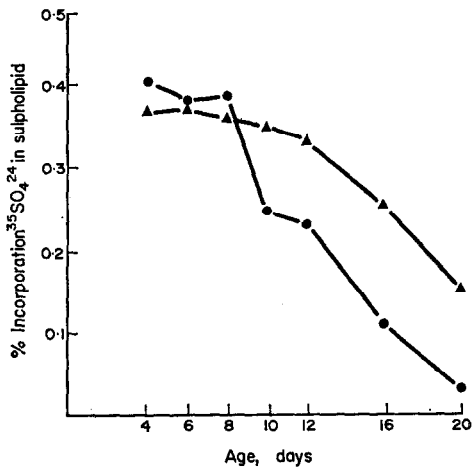

FIG. 6. THE EFFECT OF KINETIN ON $^{35}SO_4^{2-}$ INCORPORATION IN SULPHOLIPID IN AGEING DARK-GROWN BARLEY SEEDLINGS. RESULTS EXPRESSED AS A PERCENTAGE OF THE TOTAL $^{35}SO_4^{2-}$ TAKEN UP. ●—Water controls; ▲—kinetin treated.

DISCUSSION

It is well established that kinetin treatment retards senescence in detached green leaves of a variety of species.[4–6] It is widely held that the critical action of kinetin might be the maintenance of protein synthesizing systems, perhaps by regulating RNA synthesis.[7,8] Changes in protein and nucleic acid metabolism accompany the loss of chlorophyll in ageing cucumber cotyledons[9] and Draper[10] demonstrated changes in plastid lipids in the same tissue. In barley, kinetin treatment overcomes to some extent the loss of the ability of ageing etiolated leaves to produce chlorophyll on exposure to light (Fig. 1a). This effect appears to be analogous to the situation found in detached green leaves treated with kinetin.

The rate of P_{652} regeneration (Fig. 4a) decreases before the rate of conversion of exogenous ALA to P_{632} is affected by ageing (Fig. 4d). Although ALA-dehydratase activity increases in greening systems[11,12] there is probably only a slow turnover of the enzymes catalysing the conversion of ALA to protochlorophyllide whereas the enzyme(s) responsible for ALA formation in higher plants (ALA-synthetase?)[13,14] exhibits rapid turnover. Inhibitor studies suggest that the enzyme(s) for ALA synthesis may undergo rapid destruction and require rapid protein synthesis for maintenance. Kirk,[15] on the other hand, has shown

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that protein synthesis other than that required for ALA synthesis is also necessary for chlorophyll formation in *Euglena* and suggests that this may be structural protein.

Succinyl-CoA synthetase is known to increase in etiolated barley leaves exposed to light.[16] The increase on illumination is dependent on the age of the etiolated tissue and the decline in activity with age does not appear to be affected by kinetin treatment (Fig. 5a). RNAse activity (Fig. 5b) and, to a lesser extent, chlorophyllase activity (Fig. 5c) increased in the ageing seedlings maintained in darkness, the increases in activity being suppressed by kinetin. Kinetin treatment extended the period of active protein synthesis (Fig. 1b) in ageing etiolated barley seedlings and also chlorophyll synthesis when the seedlings were exposed to light (Fig. 1a). Whether kinetin is specifically involved in maintaining active plastid protein synthesis remains to be determined.

Sodek and Wright[17] report an increase in RNAse activity expressed as units per gram fresh weight in detached green leaves of wheat and barley, but in the present investigation RNAse increases were observed only when expressed on a protein basis. Other workers[18] report a decline in RNAse activity (units/g fresh weight) in detached barley leaf segments. The RNA present in the chloroplasts of mature leaves may degrade rapidly with senescence[19] and an active RNAse component may develop in the plastid.[20] Chlorophyllase activity increases in greening systems[21,22] and it has been suggested that this may be the enzyme responsible for the phytylation of chlorophyllide.[23] In the present work, however, the slight increase in chlorophyllase in ageing etiolated tissue (Fig. 5c) might also suggest some hydrolytic role for this enzyme.

Sulpholipid appears to be closely associated with chlorophyll formation.[24–26] In the ageing etiolated seedlings the incorporation of $^{35}SO_4^{2-}$ into sulpholipid is reduced (Fig. 6) as is the ability to regenerate P_{652} (Fig. 4a). Thus for sulpholipid synthesis there is probably a requirement for active protein synthesis either structural or enzymic.

EXPERIMENTAL

Plant material. Barley seeds (*Hordeum vulgare* L. cv. Proctor) were grown in the dark in moist vermiculite at 24°. Selected pots of seedlings were sprayed daily with 100 ml 0·16 mM solution of kinetin (6-furfurylaminopurine, Sigma). Control seedlings were sprayed with distilled water. Seedlings were taken for experimental purposes at regular intervals from 4 to 20 days after sowing. Light treatment was provided from fluorescent tubes (6000 lx).

Enzyme assays. ALA-dehydratase (E.C. 4.2.1.24) was assayed, in buffered extracts prepared from acetone powders of the plant material.[27] Activity is expressed as μmoles porphobilinogen formed/mg protein/10 min. Succinyl-CoA synthetase (E.C. 6.2.1.5) was assayed in extracts of acetone powders.[28] Specific activity is expressed as μmoles hydroxamic acid produced/mg protein/60 min. Chlorophyllase (E.C. 3.1.1.14) activity was determined by following the breakdown of phaeophytin *a* by acetone powder

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preparations in the presence of Triton X-100.[29] Results are expressed as μg phaeophytin hydrolysed/100 mg acetone powder 60 min. RNAse activity was determined by the method of Sodek and Wright.[17] Specific activity is given as μmoles acid-soluble nucleotides produced/mg protein/60 min. Acetone powders were prepared by the method of Loomis.[30] Soluble protein was determined using Folin–Ciocalteu reagent.[31] Chlorophyll was determined in 80% acetone.[32] Protochlorophyllide and protochlorophyll in 80% acetone extracts of etiolated leaves were separated from each other by phase separation against light petroleum (b.p. 60°–80°) when the protochlorophyll largely entered the ether phase. Quantitative determinations were made spectrophotometrically.[33]

In vivo *studies*. The apical 5 cm of each leaf were removed and placed vertically in glass cells with a light path of 2 mm. Usually 10 such leaves were necessary to fill a cell 50 × 10 × 2 mm and to give a layer 1 leaf thick. The samples were scanned in the second cell holder of an S.P. 800 Unicam recording spectrophotometer. Endogenous protochlorophyllide was removed by light treatment and its regeneration recorded at 652 nm using an expanded scale unit. ALA-induced protochlorophyllide synthesis was measured by adding 1·5 ml 0·01 M ALA solution, pH 6·9, to the cell and following the increase in absorption at 632 nm. For the determination of sulpholipid formation $^{35}SO_4^{2-}$ (carrier free) was obtained from the Radiochemical Centre, Amersham. 30 seedlings were cut off at the base and placed in 30 ml distilled water containing 50 μCi $^{35}SO_4^{2-}$. The plants were kept in the dark in a current of air to facilitate uptake. After all the radioactive solution had been taken up, 10 ml distilled water was added and the lipids were extracted from the seedlings when this water had been taken up. Sulpholipid was extracted and purified by TLC.[34] Areas on the chromatograms corresponding to sulpholipid were removed, eluted in warm ethanol, and assayed for radioactivity. Results are expressed as percentage of the total activity recovered in alcohol.

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Key Word Index—Hordeum vulgare; Gramineae; barley; chlorophyll; kinetin effect; ageing leaves.