Effect of Second-Leaf Removal or Kinetin Treatment on the Nucleic Acid Metabolism of Senescing First Seedling Leaf of Barley

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1. Changes in nucleic acid metabolism in first seedling leaves of barley plants during aging (from 7 to 27 days) were followed, and the effect of continual removal of the second leaf and basal meristem or of treating the first leaf with 20 p.p.m. kinetin on these changes was examined. During aging of the first seedling leaves the ribosomal RNA, DNA and soluble RNA declined, with ribosomal RNA showing the most rapid fall. This was, however, accompanied by increased incorporation of ^{32}P into RNA, which reached its peak on the fifteenth day. 2. Second-leaf removal partially suppressed first-leaf senescence as judged by retarded chlorophyll and nucleic acid decline and by a decreased extent of RNA labelling. Treatment with kinetin, however, did not prove effective. 3. No significant differences in the sucrose-gradient pattern of ^{32}P -labelled nucleic acids or in the ^{32}P -labelled nucleotide composition of RNA fractions during aging or during the two treatments were noted, except for a decrease in CMP content of soluble RNA during aging. 4. The results demonstrate that important changes in RNA metabolism are associated with leaf senescence.

Excised leaves floated on water senesce rapidly, this being manifested by the loss of chlorophyll, RNA and protein (Srivastava, 1967a). However, if the leaves are floated on kinetin solution the senescence is retarded (Richmond & Lang, 1957). Senescence in mature parts of the plants can also be induced or accelerated by the influence of the young growing organs (Mothes, 1960, 1961; Leopold, 1961), and the removal of younger parts of the plants (Molisch, 1929; Schumacher, 1930) or the treatment of the older parts with kinetin (Mothes, 1964) has been known to delay senescence. Kulayeva (1962) has even shown that tobacco leaf tissue that had turned partly yellow could be rejuvenated by kinetin treatment, a result implying that the senescent processes were reversible. Most of our knowledge of the biochemistry of plant senescence (Srivastava, 1967a) has come from studies on isolated tissues and only a little is known (e.g. Cherry, Chroboczek, Carpenter & Richmond, 1965) about the biochemical changes that occur in intact senescing organs and the effects of senescence-retarding treatments on these changes. Since nucleic acid metabolism is of key importance (Osborne, 1965; Srivastava, 1967a) in senescence, we have studied the changes with age in the amount and the extent of incorporation of ^{32}P into nucleic acids in first seedling leaves of barley, and the

effect of second-leaf removal or treatment with kinetin on these changes.

MATERIALS AND METHODS

Growth of plants. Barley (*Hordeum vulgare* L. var. Wolfe) plants were grown in soil in pots on greenhouse benches and given 16 hr. of light/day. The experiment was started when the plants were 7 days old and the treatments were started on the eleventh day. After 11 days' growth plants were divided into three batches. The first group was allowed to continue normal growth. In the second group, the second leaf was pulled out and any growth of the second leaf that occurred due to the persistence of the basal meristem was removed every alternate day up to and including day 27. In the third group of plants, the first seedling leaf was treated on the eleventh day and every alternate day thereafter with kinetin (20 p.p.m. in 0.01% Tween 20); a soft applicator was used to reduce mechanical damage to the leaf. The above three batches of plants are designated in the following text 'intact' plants, plants with 'second leaf removed' and 'kinetin-treated' plants respectively.

Labelling of nucleic acid with ^{32}P . The first seedling leaves from plants of different ages and treatments were sterilized by 10% (v/v) Clorox for 3.5 min. and rinsed extensively with sterile water. Leaf sections (0.5 cm.) from 3 g. of leaves were repeatedly infiltrated for 20 min. in 40 ml. of the sterile incubation medium ($100\mu\text{C}$ of $\text{Na}_2\text{H}^{32}\text{PO}_4$ in 40 ml. of 0.1 M-ammonium citrate, pH 6.0) and subsequently incubated on a shaker at 25° for 100 or 220 min. After the incubation with ^{32}P , portions of the incubation medium

and of the leaf tissue (thoroughly triturated in a sterile mortar) were plated on 5.3% Bacto brainveal agar (Difco) by standard techniques; bacterial counts were noted after 72 hr. incubation at room temperature.

Isolation and fractionation of nucleic acids. The nucleic acids were isolated by a phenol-sodium dodecyl sulphate-bentonite procedure and were fractionated by sucrose-gradient centrifugation as described by Srivastava (1967b). After the extinction of each fraction had been read, the nucleic acids were precipitated by trichloroacetic acid. The RNA precipitates were collected on B₃ membrane filters and counted for ³²P in a Nuclear-Chicago series 720 counter. The details of the above procedure have been given by Srivastava (1967b).

Determination of ³²P-labelled nucleotide composition of nucleic acid fractions. After being counted, the membrane disks containing fractions 2-12, 13-20, 21-27 and 37-44 from the sucrose gradients were pooled to give samples referred to below as RNA fractions A, B, C and D respectively. Carrier yeast RNA was added to the pooled samples and, after the removal of the membrane filters with acetone, the RNA was hydrolysed with 0.3 N-NaOH for 16 hr. at 37°. After the removal of Na⁺ ions by Dowex 50 (H⁺ form), the nucleotides were separated by paper electrophoresis at pH 3.5 and located by ultraviolet light. The zones containing the nucleotides were excised and counted (Srivastava, 1967b) in a scintillation counter.

Chlorophyll and phosphorus determinations. The chlorophyll was extracted with boiling 80% ethanol and *E*₆₆₅ of the extract was determined.

Inorganic phosphorus and total phosphorus soluble in cold 5% trichloroacetic acid were measured by the procedure of Allen (1940). The amount of bound phosphorus soluble in 5% trichloroacetic acid was determined by the difference.

RESULTS AND DISCUSSION

Changes in chlorophyll and nucleic acid content. The chlorophyll content of the first seedling leaf of barley declined with age (Fig. 1) and the removal of the second leaf from the plant significantly (degrees of freedom = 4; *t* = 2.8) retarded this decline. Kinetin treatment of the first leaf, however, had no significant effect on the chlorophyll loss, in contrast with the chlorophyll-preserving effect of kinetin in excised leaves (Osborne, 1962; Srivastava, 1967a). During aging of the first leaf the total nucleic acid content, especially that of r-RNA,* showed a sharp decline from 7 to 11 days followed by a slow decline, whereas the declines in the amount of DNA and

* Abbreviations: r-RNA, ribosomal RNA; s-RNA, soluble RNA.

Fig. 1. Changes with age in chlorophyll, total nucleic acid, r-RNA₂, r-RNA₁, DNA and s-RNA content of first seedling leaves of barley from intact plants (○), from plants with the second leaf removed (●) and from plants in which the first leaf had been treated with kinetin (△). The arrows indicate the leaf age when second-leaf removal and kinetin treatments began.

Table 1. Changes in total nucleic acid and ^{32}P incorporation in first seedling leaves of barley under various treatments

Values are derived from data as of Fig. 3. Results in parentheses are for 2 hr. incubation with ^{32}P . The specific radioactivity is determined by dividing the total radioactivity by the E_{260}^{cm} value, which measures total nucleic acid concentration.

Leaf age (days)	Intact plant			Second leaf removed			Kinetin-treated		
	$E_{260}^{\text{cm}}/\text{g.}$ of tissue	Radioactivity (counts/min./g. of tissue)	Sp. radioactivity	$E_{260}^{\text{cm}}/\text{g.}$ of tissue	Radioactivity (counts/min./g. of tissue)	Sp. radioactivity	$E_{260}^{\text{cm}}/\text{g.}$ of tissue	Radioactivity (counts/min./g. of tissue)	Sp. radioactivity
7	24.9 (28.2)	60577 (21592)	2434 (765)						
9	19.4 (18.2)	78019 (23127)	4016 (1271)						
11	14.9 (13.4)	187145 (42626)	12574 (3188)						
13	9.3	227202	24334	10.2	185516	18182	13.6	280732	20546
15	9.8	430771	44031	10.9	200806	18378	11.4	288811	25194
19	7.6	406932	53403	12.3	229694	18604	8.3	412822	49818
23	4.6	292970	63505	4.8	233036	47947	6.9	165392	24028
27	4.0	57822	14479	5.1	55350	10867	3.1	162744	57719

s-RNA were much slower. Since a similar loss of nucleic acids with age was found when the leaves were analysed directly for nucleic acids, the loss of nucleic acids with age noted in Fig. 1 cannot be ascribed to the degradative effect of nucleases during the incubation with ^{32}P . Further, declines in the concentrations of nucleic acids as presented in Fig. 1 have also been noted during the senescence of peanut cotyledons (Cherry *et al.* 1965) and of excised pea leaves (Wood & Bradbeer, 1965). The decline in the amount of nucleic acids during senescence of the first seedling leaf of barley may result from the export of material from the first leaf to the growing second leaf, so that the excision of the second leaf should retard the decline of nucleic acids from the first leaf. Although the effects of second-leaf removal or the kinetin treatment on the amount of total nucleic acid or nucleic acid fractions (Fig. 1) were not significant at the 5% level in the paired *t* test, the failure to reach significance with total nucleic acids and r-RNA may have occurred because the treatments were not equally effective throughout the duration of the treatments. For example, if the results for the nineteenth day were excluded the effect of second-leaf removal on total nucleic acid content became significant at the 1% level, and if the results for the twenty-seventh day were excluded the effect of kinetin on r-RNA₁ (light r-RNA) (Srivastava, 1967b) became significant and its effect on r-RNA₂ (heavy r-RNA) and total nucleic acid came very close to being significant. It may therefore be concluded that although kinetin treatment or second-leaf removal did not prove successful in eliminating the effect of the competing meristem, both treatments did appear to suppress the loss of nucleic acids from the first leaf.

Changes in the amount of ^{32}P incorporation into nucleic acids. While the amount of nucleic acids was declining, the extent of ^{32}P incorporation into the nucleic acids of first seedling leaves increased markedly with age, reaching a maximum on the fifteenth day, and even on the twenty-seventh day, when the leaves were in an advanced stage of senescence, the incorporation was as high as on the seventh day (Table 1). The decline in the amount of nucleic acids and the increase in the amount of ^{32}P incorporation resulted in a tremendous increase in the specific radioactivity of the nucleic acids, which at its peak was 26 times that on the seventh day. The non-linear incorporation of ^{32}P observed on the seventh day was also present on the ninth and eleventh days (Table 1).

During the senescence of the first seedling leaf the amounts of both inorganic phosphorus and cold 5% trichloroacetic acid-soluble bound phosphorus declined (Table 2) and thereby caused a decrease in the phosphorus pools of the tissue. The increase in RNA labelling during the senescence of the first

leaf may therefore have resulted from a decline in phosphorus pools and from changes in permeability, although other factors may also have been involved.

It is noteworthy (Table 1) that the removal of the second leaf on the eleventh day significantly retarded the increase of incorporation of ^{32}P into nucleic acids, whereas the kinetin treatment showed an erratic effect on ^{32}P incorporation, ranging from

higher incorporation on the thirteenth and twenty-seventh days to inhibition on the fifteenth and twenty-third days.

The effect of second-leaf detachment and the kinetin treatment on the labelling of different RNA fractions from the first leaf presented in Fig. 2 show that the inhibiting effect of leaf detachment on the labelling of RNA fractions *A*, *B* and *C* was significant (at the 5% level), but for RNA fraction *D* it fell slightly short of significance. The changes in the labelling of RNA fractions due to kinetin, however, did not reach the level of significance for any fraction. Nevertheless, the suppression of labelling of RNA fractions *B* and *D* by kinetin during the period of the treatments when leaf detachment had the greatest effect is noteworthy.

Changes in sedimentation profile of nucleic acids.

As the ^{32}P and extinction sedimentation profiles of nucleic acids from leaves of intact plants, kinetin-treated or second-leaf-removed plants were very similar, the nucleic acid profiles of 7, 11, 15 and 27 day-old leaves from intact plants only are presented in Fig. 3. The profiles show four extinction peaks corresponding to heavy r-RNA (r-RNA₂), light r-RNA (r-RNA₁), DNA and s-RNA. The radioactivity profiles in Fig. 3 do not follow the extinction peaks, indicating the heterogeneous nature of the labelled RNA. Noticeable is the absence from Fig. 3 of any DNA radioactivity peaks

Table 2. *Changes in inorganic phosphorus and cold-5%-trichloroacetic acid-soluble bound phosphorus content of aging first seedling leaves of barley from intact plants*

Results are the average of two experiments.

Leaf age (days)	Cold-5%-trichloroacetic acid-soluble P ($\mu\text{g. of P/g. fresh wt.}$)	
	Inorganic P	Bound P
7	131.6	47.4
9	111.5	44.8
11	98.2	41.8
13	103.2	34.0
15	62.2	20.0
19	23.3	28.7
23	12.3	15.3
27	44.3	28.1

Fig. 2. Changes with age in the amount of ^{32}P incorporation into various RNA fractions from first seedling leaves of barley from intact plants (○), from plants with the second leaf removed (●) and from plants in which the first leaf had been treated with kinetin (△). The arrow indicates the leaf age when second-leaf removal and kinetin treatments began. RNA fractions *A*, *B*, *C* and *D* represent tubes 2–12, 13–20, 21–27 and 37–44 respectively of the sucrose gradients as presented in Fig. 3, and correspond to messenger RNA, r-RNA₂, r-RNA₁ and s-RNA.

Fig. 3. Changes with age in the sedimentation profile of nucleic acids from first seedling leaves of barley from intact plants. Leaf segments (3 g.) were incubated with ^{32}P for 4 hr. Nucleic acids were sedimented for 15 hr. at 25 000 rev./min. in a SW 25.1 rotor of a Spincro model L ultracentrifuge through a 5–20% linear sucrose gradient containing 0.05 M-sodium phosphate buffer, pH 6.8, and 1 mM-MgCl₂. Extinction (●) refers to 40-drop fractions diluted to 3 ml. with water. ^{32}P radioactivity (○) has been corrected for decay.

associated with bacterial contamination which is in agreement with the findings of Lonberg-Holm (1967). In experiments of the sort shown in Fig. 3, the bacterial counts in the total incubation medium or in the equivalent amount of fresh tissue ranged from 0 to 600 colonies, indicating that the tissue was rendered virtually free of bacteria by the Clorox treatment.

Changes in ^{32}P -labelled nucleotide composition of RNA fractions. The nucleotide composition measured by the percentage of the total ^{32}P in each nucleotide of RNA fraction A (CMP 24.0, AMP 26.5, GMP 26.9, UMP 22.6%), fraction B (CMP 22.7, AMP 28.0, GMP 26.8, UMP 22.6%), fraction C (CMP 20.5, AMP 29.9, GMP 24.0, UMP 25.6%) and fraction D (CMP 45.3, AMP 19.9, GMP 19.9, UMP 14.9%) from 7-day-old leaves showed that the RNA fractions A, B and C were more similar to DNA (A + T = 55%) than to r-RNA (AMP + UMP = 44%), whereas fraction D (s-RNA) was high in CMP content. The ^{32}P -labelled nucleotide composition of RNA fractions A, B and C changed little during aging of the first seedling leaf from the intact plants,

and kinetin treatment or second-leaf excision also did not have any effect. Only the s-RNA showed a decline in CMP content from 45% on the seventh day to 26% on the twenty-seventh day, whereas on removal of the second leaf or kinetin treatment the CMP content of the s-RNA remained at 30% and 31% respectively. The decrease in the labelling of CMP in s-RNA with age may result from changes in s-RNA itself or by contamination of s-RNA by degradation products of r-RNA.

The enhanced incorporation of ^{32}P into RNA of senescing first seedling leaves of barley observed in the present work could be explained on the basis of decline in the phosphorus pool and the changes in permeability of the tissue, although other factors may also be involved. Although kinetin treatment of the first leaf or the removal of the second leaf could not overcome the effect of competing meristem in barley (a monocotyledonous plant), the latter treatment especially suppressed to some extent the changes occurring during the senescence of the first leaf. The present methods of analysis, however, failed to show any differences in the pattern of

labelling or in the nucleotide composition of RNA during senescence or during the treatments given. At present it is therefore a matter of speculation whether senescence involves synthesis of specific messenger RNA molecules responsible for the production of destructive enzymes such as ribonucleases, peptidases and cellulases, which have been shown to increase during aging (Hanson, Wilson, Chrispeels, Krueger & Swanson, 1965; Cherry *et al.* 1965; Anderson & Rowan, 1966; Horton & Osborne, 1967).

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