Biochem. J. (1968) **110**, 683
Printed in Great Britain

Studies on the Chromatin of Barley Leaves during Senescence

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(*Received* 13 *May* 1968)

1. The activity of soluble ribonuclease and deoxyribonuclease first declined during senescence, but later increased during advanced stages of senescence. 2. Young leaves had very low ribonuclease or deoxyribonuclease activity associated with the chromatin, but the activity of these enzymes increased progressively during senescence until the leaves died. 3. No significant changes in the composition of chromatin from first seedling leaves of barley plants during aging (from 7 to 25 days) were noted. 4. The amount of RNA synthesized by chromatin *in vitro* declined as the leaf aged. However, if the loss of RNA due to chromatin-associated ribonuclease was taken into account, the RNA-synthesizing activity of chromatin from senescing (15–16-day-old) leaves appeared to be somewhat higher than that of chromatin from young (7–8-day-old) leaves. In leaves at the terminal stages of senescence (23 days old) the estimates of RNA synthesis by chromatin could not be made owing to complications created by high nuclease activities. 5. It is suggested that senescence may be triggered by a decline in some hormonal factor in leaves, and that the resulting production of chromatin-associated deoxyribonuclease and ribonuclease in increasing proportions may progressively cause increased degradation of DNA and newly synthesized RNA, so that ultimately the cellular functions are impaired and the cells die.

Excised leaves floated on water senesce rapidly (Srivastava, 1967). In the barley the first seedling leaf is also induced to senesce when the plant is about 7 days old and the second leaf starts to grow. Senescence in excised or intact leaf is a gradual process that is characterized by a continuous decline in the amount of chlorophyll, nucleic acids and proteins and by alterations in the rate of incorporation of precursors into nucleic acids and proteins (Srivastava & Atkin, 1968; Atkin & Srivastava, 1968, and unpublished work). Since these studies as well as others (see review by Srivastava, 1967) suggested that senescence may involve changes at the nuclear-gene level the composition and the template activity of the chromatin from young and senescing leaves was determined. In view of the finding of nuclease activity associated with chromatin that increased during senescence, the activities of soluble nucleases were also determined. The results of these experiments are described in this paper.

MATERIALS AND METHODS

General. Barley (*Hordeum vulgare* L. var. Wolfe) plants were grown in soil in pots on greenhouse benches and given 16hr. of light/day. The first seedling leaves from plants of different ages were used for the preparation of chromatin and for other determinations. During senescence the whole leaf lost chlorophyll uniformly with some accelerated loss at the tip so that in 25-day-old leaves one-quarter to one-third of the leaf at the tip had dried and was therefore discarded. Except for these very old leaves the fresh weight per leaf (100–110mg.) did not differ significantly in leaves of various ages.

Preparation of chromatin and DNA. The chromatin (from 30–40g. of leaves) was prepared and purified by ultracentrifugation at 70000*g* for 2hr. through 1·7M-sucrose essentially as described by Huang & Bonner (1962). The transparent chromatin pellet obtained by ultracentrifugation was washed by suspending in 0·05M-tris–HCl buffer, pH8·0, containing 0·01M-β-mercaptoethanol and centrifuging at 30000*g* for 30min. The washed chromatin pellet was dispersed in 0·01M-tris–HCl buffer, pH8·0, and dialysed overnight against the same buffer at 1°. After dialysis the chromatin (30ml.) was used in different determinations. The DNA from chromatin was prepared by the procedure of Marmur (1961).

Determination of template activity. The template activity of chromatin was determined as described in the legend to Table 2. The composition of the assay mixture was that of Chamberlin & Berg (1962) and the DEAE-cellulose-purified RNA polymerase from *Escherichia coli* used in the assays was also prepared by their procedure. Since 23-day-old leaves already represented terminal stages of senescence the chromatin from leaves older than 23 days was not used in the template assay.

Chemical analysis of chromatin. The histones from chromatin were extracted with 0·5 N-HCl and estimated by the procedure of Lowry, Rosebrough, Farr & Randall (1951). The residue left after HCl extraction was completely solubilized in 0·3 N-NaOH. The protein estimation on a sample of the NaOH solution gave the amount of residual protein. Another sample of the NaOH solution was made 5% (w/v) with respect to trichloroacetic acid, heated at 90° for 15 min. and centrifuged. DNA and RNA were estimated on the supernatant thus obtained by using the diphenylamine (Burton, 1956) and orcinol (Markham, 1955) procedures respectively.

Preparation of soluble nuclease extracts. A 1 g. sample of leaves was homogenized with 0·05 M-potassium acetate buffer, pH 6·5, and acid-washed sand in a chilled pestle and mortar and the homogenate was centrifuged at 30000*g* for 30 min. at 0°. The supernatant obtained was made up to 25 ml. with buffer and samples were used for the assay of soluble ribonuclease and deoxyribonuclease activity.

Assay of ribonuclease and deoxyribonuclease. For the estimation of chromatin-associated ribonuclease 1–2 ml. of chromatin preparation was incubated with 2 ml. of 0·1% *Torula* s-RNA (transfer RNA) (Calbiochem, Los Angeles, Calif., U.S.A.) in 0·05 M-tris–HCl buffer, pH 7·5, for 0, 1, 2 and 4 hr. at 40°. For soluble ribonuclease determinations 2 ml. of enzyme preparation was incubated with 2 ml. of 0·5% *Torula* s-RNA in 0·05 M-potassium acetate buffer, pH 6·0, for 0 and 2 hr. at 40°. For the estimation of chromatin-associated deoxyribonuclease 1–2 ml. of chromatin preparation was incubated with 2 ml. of 0·1% DNA (sodium salt) (Calbiochem), 2 ml. of 0·05 M-tris–HCl buffer, pH 7·5, and 0·1 ml. of 0·4% $MgCl_2$ for 0, 1, 2 and 4 hr. at 40°. For the assay of soluble deoxyribonuclease 4 ml. of enzyme preparation was incubated with 2 ml. of 0·1% DNA (sodium salt) in 0·05 M-potassium acetate buffer, pH 6·0, and 1 ml. of 0·5% $MgCl_2$ for 0 and 2 hr. at 40°. Where the effect of pH on the activity of chromatin-associated nucleases was to be determined the 0·05 M-tris–HCl buffer of appropriate pH was substituted for the pH 7·5 buffer. In experiments where the effect of pH on the activities of soluble nucleases was to be determined the enzymes were extracted with water and the 0·05 M-tris–HCl or potassium acetate buffer of appropriate pH was used in the assay mixture.

At the end of the incubation time the undigested nucleic acid was precipitated with 15 ml. of chilled 5% (w/v) $HClO_4$ in ethanol. The contents were chilled at −20° for 3 hr. and then centrifuged at 30000*g* for 20 min. The E_{260} values of supernatants (made up to 100 ml. with water for soluble nucleases) for 1, 2 or 4 hr. incubations were read against a blank (for zero time). Ribonuclease or deoxyribonuclease activity was expressed as enzyme units/mg. of DNA in chromatin for chromatin-associated nucleases and as enzyme units/g. fresh wt. of leaves for soluble nucleases. One enzyme unit was defined as an increase of 1·0 E_{260} unit in 1 ml. of solution for an incubation period of 1 hr. Under the conditions of the assay the rates of the enzyme reactions were linear with time.

RESULTS AND DISCUSSION

Soluble and chromatin-associated nuclease activities. The results presented in Fig. 1 show that the activities of soluble nucleases decreased during the early stages of senescence and that it was only in the advanced stages of senescence that these activities increased. Throughout senescence, however, the amounts of RNA, DNA and chlorophyll continued to decline. For example, the RNA, DNA and chlorophyll contents of the 15-day-old leaves were 36, 70 and 40% respectively and of 25-day-old leaves 13, 40 and 17% respectively of the values obtained with 7-day-old leaves (for detailed analysis

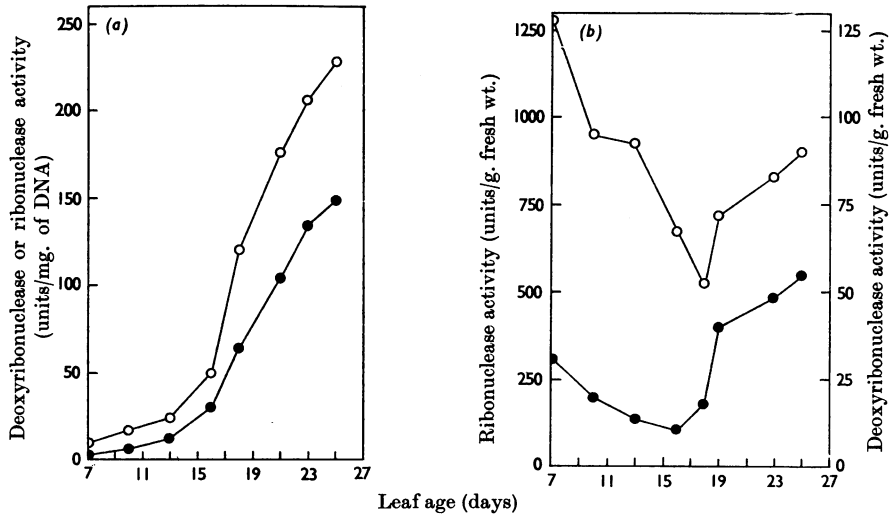

Fig. 1. Changes in chromatin-associated (*a*) and soluble (*b*) deoxyribonuclease (●) and ribonuclease (○) activities of barley leaves during senescence. Experimental details are given in the text.

of 7–25-day-old leaves see Srivastava & Atkin, 1968). In contrast with soluble nucleases the activities of chromatin-associated nucleases (Fig. 1*a*) in 7-day-old leaves were very low and as the leaves aged these activities continued to increase. Since nuclease activities in chromatin from senescent leaves in the presence of chromatin from 7-day-old leaves were simply additive, the consistently low nuclease activities of chromatin from young leaves could not have been due to the presence of nuclease inhibitors.

The question whether the 'chromatin-associated nucleases' were indeed associated with chromatin *in vivo* and were different from soluble nucleases deserves some comment. The isolation procedure (Huang & Bonner, 1962) for the preparation of chromatin is designed to exclude contamination from soluble enzymes or cellular organelles. The pH optima of soluble ribonuclease and deoxyribonuclease were 6·0 and 5·5 respectively, whereas the pH optima of chromatin-associated ribonuclease and deoxyribonuclease were 7·0 and 7·5 respectively. Further, both ribonuclease (Marushige & Bonner, 1966) and neutral deoxyribonuclease (Swingle, Cole & Bailey, 1967) have been found associated with chromatin from mammalian cells. It may therefore be concluded that the nuclease activities associated with chromatin from barley leaves are real and do not represent a contamination. It is noteworthy that 97% of the activity of both chromatin-associated nucleases was destroyed in 10min. at 100°.

Composition and template activity of chromatin. The composition of barley leaf chromatin presented in Table 1 resembles the composition of chromatin from other sources (Huang & Bonner, 1962; Dingman & Sporn, 1964).

During leaf senescence no significant change in the DNA/histone ratio of chromatin was noted, although the chromatin from leaves in an advanced stage of senescence (23–25 days old) appeared to be low in residual protein and high in RNA. The results in Table 2 show that the RNA-synthesizing activity of chromatin from leaves of different ages was limited by the amount of RNA polymerase.

Table 1. *Composition of chromatin from first seedling leaves of barley of diffent ages*

Results for 7-day-old leaves are given as means ± S.D. for five determinations.

Leaf age (days)	Chromatin components (% of total mass) DNA	Histone	Residual protein	RNA
7	36·2 ± 1·0	35·4 ± 2·5	20·3 ± 2·7	8·2 ± 1·8
10	33·7	31·2	22·5	12·5
13	33·5	32·4	21·1	12·9
16	34·5	30·4	20·3	14·8
18	34·7	31·6	22·5	11·1
21	33·9	33·0	20·9	12·2
23	35·5	35·4	12·9	16·1
25	32·7	33·6	12·9	20·7

Table 2. *Activity of chromatin from first seedling leaves of barley of different ages in DNA-dependent RNA synthesis*

The complete incubation mixture (1ml.) contained: tris–HCl buffer, pH 8·0, 40μmoles; $MnCl_2$, 1μmole; $MgCl_2$, 4μmoles; CTP, GTP and UTP, each 0·2μmole; [8-^{14}C]ATP, 0·2μc (specific radioactivity 25μc/μmole); β-mercaptoethanol, 12μmoles; DEAE-cellulose-purified *E. coli* RNA polymerase, approx. 20μg.; chromatin, as indicated. The reaction was started by adding primer last. After 15min. incubation at 40° 40μmoles of unlabelled ATP, 1ml. of cold 25% (w/v) trichloroacetic acid and 50μg. of bovine serum albumin were added. The precipitates were collected on B_6 membrane filters (Carl Schleicher and Schuell Co., Keene, N.H., U.S.A.), washed with 5% trichloroacetic acid and counted in a scintillation counter. The results given in the Table are as follows: (*A*) chromatin (μg. of DNA equivalent); (*B*) radioactivity incorporated into RNA (counts/min.); (*C*) radioactivity incorporated into RNA (counts/min./μg. of DNA equivalent).

Leaf age	7 days			8 days			15 days			16 days			23 days		
Incubation mixture	(*A*)	(*B*)	(*C*)	(*A*)	(*B*)	(*C*)	(*A*)	(*B*)	(*C*)	(*A*)	(*B*)	(*C*)	(*A*)	(*B*)	(*C*)
Complete	2·3	1232	536	1·7	850	500	1·8	825	458	1·3	666	512	1·0	217	217
Complete	3·8	1790	471	2·9	1500	517	3·0	1265	422	2·2	994	452	1·8	344	190
Complete	7·6	2541	334	5·8	1977	341	6·0	1454	242	4·4	1039	236	3·6	618	171
Polymerase omitted	3·8	72	19	2·9	56	19	3·0	36	12	2·2	34	15	1·8	20	11
GTP and UTP omitted	3·8	80	21				3·0	110	37						
Mg^{2+} and Mn^{2+} omitted	3·8	623	164				3·0	425	142						
Actinomycin D (20μg.) added	3·8	76	20				3·0	98	33						
Pancreatic ribonuclease (20μg.) added	3·8	243	64				3·0	210	70						

Table 3. *Effect of chromatin on RNA synthesized by DNA from 7-,16- and 23-day-old barley leaves*

The composition of the incubation mixture for RNA synthesis was as given in Table 2.

Sample	Radioactivity incorporated into RNA (counts/min.)
(1) 1·5 μg. of DNA	8426
(2) 1·5 μg. of DNA + 1 μg. of chromatin from 7-day-old leaves	9026
(3) 1·5 μg. of DNA + 1 μg. of chromatin from 16-day-old leaves	8176
(4) 1·5 μg. of DNA + 1 μg. of chromatin from 23-day-old leaves	5231
(5) After RNA synthesis by 1·5 μg. of DNA the contents were incubated for 15 min. with 1 μg. of chromatin from 7-day-old leaves and 20 μg. of actinomycin D	8501
(6) Same as (5) but with chromatin from 16-day-old leaves	7949
(7) Same as (5) but with chromatin from 23-day-old leaves	4875

Further, the template activity of chromatin was dependent on the presence of all four ribonucleoside triphosphates, Mg^{2+} and Mn^{2+}, and the activity was inhibited by actinomycin D or ribonuclease. The results in Table 2 show that, if the loss of RNA by chromatin-associated ribonuclease is taken into account (Table 3), the RNA-synthesizing activity of chromatin from 15–16-day-old barley leaves may be higher than that of chromatin from 7-day-old leaves. The denatured DNA has been found to be a better template for RNA synthesis (Karkas & Chargaff, 1967; Stout & Mans, 1968) and the chromatin-associated deoxyribonuclease by its action on DNA may to some extent increase the template activity of chromatin. In leaves at the terminal stages of senescence (such as 23-day-old leaves), which have high nuclease activities, the estimates of RNA degraded (during RNA synthesis by chromatin) may be difficult to estimate from the results in Table 3 owing to variable amounts of complex-formation between RNA and DNA denatured by deoxyribonuclease. Such complexes are known to be resistant to ribonuclease (Stout & Mans, 1968). Further, Table 3 does not give any information about the further damage to the template DNA by deoxyribonuclease that may occur during warming.

The results presented in this paper suggest that during senescence of barley leaves the production of chromatin-associated deoxyribonuclease and ribonuclease is increased and that this progressively causes increased degradation of DNA and newly synthesized RNA, so that ultimately the cellular functions are impaired and the cells die. Decline in some hormonal factor may be responsible for triggering the senescence, since kinetin both retarded senescence and also suppressed drastically increases in the chromatin-associated nucleases in excised leaves (Srivastava, 1968).

This investigation was supported by National Science Foundation Grant GB-4720X and U.S. Atomic Energy Commission Contract AT(30-1)3722.

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