Plant Physiol. (1977) 60, 182-189

Gibberellic Acid Enhancement of DNA Turnover in Barley Aleurone Cells[1]

Received for publication September 29, 1976 and in revised form January 16, 1977

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ABSTRACT

When imbibed, deembryonated halfseeds from barley (*Hordeum vulgare* L., var. Himalaya) are incubated in buffer, the DNA content of the aleurone layer increases 25 to 40% over a 24-hour period. In contrast, the DNA of isolated aleurone layers declines by 20% over the same time period. Gibberellic acid (GA) causes a reduction in DNA levels in both halfseed aleurone layers and isolated aleurone layers. GA also increases the specific radioactivity of [^{3}H]thymidine-labeled halfseed aleurone layer DNA during the first 12 hours of treatment. Pulse-chase studies demonstrated that the newly synthesized DNA is metabolically labile.

The buoyant density on CsCl density gradients of hormone-treated aleurone DNA is identical with that of DNA extracted from whole seedlings. After density-labeling halfseed DNA with 5-bromodeoxyuridine, a bimodal absorption profile is obtained in neutral CsCl. The light band (1.70 g/ml) corresponds to unsubstituted DNA, while the heavy band (1.725-1.74 g/ml) corresponds to a hybrid density-labeled species. GA increases the relative amount of the heavy (hybrid) peak in halfseed aleurone layer DNA, further suggesting that the hormone enhances semiconservative replication in halfseeds.

DNA methylation was also demonstrated. Over 60% of the radioactivity from [^{3}H-Me]methionine is incorporated into 5-methylcytosine. GA has no effect on the percentage distribution of label among the bases.

It was concluded that GA enhances the rate of DNA degradation and DNA synthesis (turnover) in halfseeds, but primarily DNA degradation in isolated aleurone layers. Incorporation by isolated aleurone layers is due to DNA repair. Semiconservative replication apparently plays no physiological role in the hormone response, since both isolated aleurone layers and gamma-irradiated halfseeds respond normally. The hypothesis was advanced that endoreduplication and DNA degradation are means by which the seed stores and mobilizes deoxyribonucleotides for the embryo during germination.

The aleurone layer of barley karyopses consists of nondividing, protein-storing secretory cells which synthesize and release α-amylase and other hydrolases in response to gibberellic acid (17). The primary function of the tissue is to mobilize the total food reserves of the endosperm for the growing embryo during germination. GA stimulation of the activity of α-amylase, protease, ribonuclease, and β-1,3-glucanase has been shown to be due to *de novo* synthesis (17). GA also increases the synthesis of poly(A)RNA, as well as the level of translatable α-amylase messenger RNA, within 4 hr of treatment (12, 14). It thus appears that the hormone acts at the level of transcription, although studies on hormone-induced RER synthesis indicate that translation may also be affected (7, 16).

The role of DNA metabolism in aleurone cells has not been investigated in detail. The tissue has often been described as triploid, derived from the primary endosperm nucleus, but no direct measurements of aleurone cell nuclear DNA have been made until recently. Maherchandani and Naylor (23) analyzed the nuclear DNA content of differentiating aleurone cells of *Avena fatua* by microspectrophotometry of Feulgen-stained nuclei and reported deviations from DNA constancy. Immature aleurone cells were initially 3C and 6C, whereas mature aleurone cells in the dry seed were distributed over a range from ~2 C to ~7 C. It was speculated that unscheduled DNA synthesis, possibly gene amplification, in combination with DNA degradation could account for the alterations in nuclear DNA during maturation.

A similar study has now been carried out on developing barley aleurone layers by Keown (20). As in *Avena*, barley aleurone cells are highly heterogeneous with respect to DNA content. Unlike *Avena*, at least one major peak is observed at every stage of development. These peaks gradually shift to higher DNA values during cell maturation. Thus, fully differentiated aleurone cells have major peaks in the 9 C and 12 C category, while the population of cells as a whole ranges from 2 C to 22 C.

The intriguing possibility that DNA metabolism may be a critical factor in the tissue's response to GA led us to investigate this process further. We have found that the hormone simultaneously stimulates both DNA synthesis and DNA degradation in aleurone cells, resulting in enhanced rates of DNA turnover. In agreement with others (9, 13), our results tend to rule against a physiological role for DNA metabolism. A more probable function seems to be the storage and mobilization of deoxynucleotides for use by the embryo. Preliminary reports on a portion of this work have appeared elsewhere (31, 32).

MATERIALS AND METHODS

Preparation and Treatment of Halfseeds and Aleurone Layers. Barley (*Hordeum vulgare* L. var. Himalaya) halfseeds were prepared by the procedure of Chrispeels and Varner (5). In some cases, halfseeds were imbibed in 10 mM succinate buffer (pH 5.2) as described by Firn and Kende (8). Imbibition on succinate buffer appeared to stimulate DNA synthesis.

Density labeling with BUDR[4] was performed as described by Haut and Taylor for bean roots (11). Halfseeds were imbibed in a solution containing 0.1 mM BUDR, 1 μM aminopterin, and 50 μM adenosine, glycine, and hypoxanthine. Aminopterin, a folic acid antagonist, blocked thymidylate synthesis and maximized incorporation of the density label. Halfseeds imbibed in this way

[1] This research was supported in part by National Science Foundation Grant BMS75-03391 and by a grant from the Committee on Research, University of California, Santa Cruz, to L. T.

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[4] Abbreviation: BUDR: 5-bromodeoxyuridine.

responded normally to GA. Density labeling was also achieved by imbibing halfseeds in a mixture of ^{15}N-bases (10 mM) obtained by acid hydrolysis of DNA purified from *Escherichia coli* which had been grown on $^{15}NH_4Cl$ as a nitrogen source.

The incubation medium contained either 1 mM sodium acetate buffer (pH 4.8) plus 10 mM $CaCl_2$, or 10 mM sodium succinate buffer (pH 5.2) plus 10 mM $CaCl_2$. Chloramphenicol (8 μg/ml) was added to prevent bacterial growth. All incubations were performed on a water bath shaker at 30 C. Labeled precursors were added as described in the figure legends.

γ-Treated dry seeds were irradiated from a ^{60}Co source.

DNA Extraction. Aleurone layers were rinsed repeatedly in sterile, distilled H_2O, blotted dry on a paper towel, quick frozen with liquid N_2, and lyophilized. When more than 25 aleurone layers/treatment were involved, the frozen tissue was ground to a powder in a mortar prior to lyophilization. Acid-precipitable DNA was extracted by two different procedures, both yielding similar results. In the first case, the lyophilized tissue was homogenized in a glass homogenizer with 5 ml cold 1 M NaCl-0.1 M K-phosphate buffer (pH 7.2). The homogenate was centrifuged (1000g, 5 min) to remove cell wall material, and the supernatant was decanted into a 40-ml centrifuge tube at 4 C. After removing an aliquot for uptake determinations, 5 ml of cold 20% trichloroacetic acid was added. The precipitate was collected by centrifugation and fractionated by the Schmidt-Thannhauser-Schneider procedure as described by Volkin and Cohn (34). In counting experiments, the extract was neutralized with KOH, and an aliquot was added to 10 ml Aquasol in a scintillation vial.

The second method included the one-step nucleic acid extraction procedure reported by Laulhere and Rozier (21). The nucleic acid precipitate was collected by centrifugation, drained dry, and then processed by the Schmidt-Thannhauser-Schneider procedure.

DNA Purification and Centrifugation on CsCl. DNA was purified for centrifugation on CsCl density gradients either by the Marmur procedure (24), with the addition of 1% SDS (w/v) in the grinding buffer, or by a modification of the Laulhere and Rozier one-step extraction (21). All precipitates were collected by centrifugation since aleurone DNA is granular rather than fibrous. The pellet obtained after precipitation with isopropyl alcohol was resuspended in grinding buffer minus SDS and treated with purified RNAse (200 μg/ml) (Calbiochem) for 4 hr at 37 C. The digest was deproteinated as described above. The preparation was precipitated with 2.5 volumes of ethyl alcohol and redissolved in 0.05 M acetate buffer (pH 4.8). Purified α-amylase (Calbiochem) and invertase (Calbiochem) were added at 100 μg/ml and digestion was allowed to proceed at 37 C for 4 hr. Both enzymes were pretreated at 45 C for 4 hr prior to addition to the DNA preparation to inactivate any DNAse present. The final digest was deproteinated and dialyzed overnight at 4 C against 100 volumes grinding buffer minus SDS.

We currently favor the Laulhere and Rozier procedure (21) because of its simplicity, for the initial extraction of nucleic acids, followed by treatment with RNAse and α-amylase as described for the Marmur procedure (24). The A_{260}/A_{280} ratio for either method ranged between 1.8 and 2.

The final DNA pellet was redissolved in 3 ml buffer (0.01 M tris-HCl [pH 7.6], 0.01 M EDTA, 0.1 M NaCl, 0.01% [v/v] Triton X-100) and CsCl was added to give a final density of 1.70 or 1.73 g/ml (density labeling experiments). The samples were centrifuged for 48 to 96 hr in an SW 50.1 rotor at 24 C. Fractions were obtained by puncturing the bottom of the tube and collecting the drops. Each fraction was then diluted with 1 ml H_2O, after reading the density of selected tubes, and the A_{260} was determined. An aliquot from each was then added directly to 10 ml Aquasol for counting.

Carbohydrate Assay. Carbohydrate was determined by the anthrone method (15).

DNA Assays. DNA was assayed by the diphenylamine test (6) or by the *p*-nitrophenylhydrazine reaction (35). Although both assays gave similar results, the *p*-nitrophenylhydrazine method was less subject to interference by other compounds and was therefore the method of choice.

Analysis of Methylated Bases. DNA was purified from aleurone layers which had been incubated in [^{3}H-Me]methionine. Salmon sperm DNA (1 mg) was added as a carrier. After centrifugation on a CsCl density gradient, the total DNA peak was collected and dialyzed overnight against 0.01 M sodium acetate at 4 C, and then precipitated with an equal volume of cold 20% trichloroacetic acid. The trichloroacetic acid precipitate was hydrolyzed with 70% perchloric acid (0.2 ml/mg DNA) at 100 C for 1 hr. The hydrolysate was cooled and diluted with 4 volumes of H_2O. After neutralization with 11.65 N KOH, the partially desalted hydrolysate was subjected to two-dimensional ascending paper chromatography on Whatman No. 1 paper. The first solvent was methanol-HCl-H_2O (7:2:1) and the second solvent was 1-butanol-methanol-H_2O-ammonium hydroxide (6:2:2:0.1). A mixture of authentic methylated bases was cochromatographed with the sample. The separated bases were located under short wavelength UV light, and the radioactivity was determined by cutting out each spot and counting it directly in a toluene-based scintillation fluid.

Enzymes Assays. α-Amylase was assayed by the method of Jones and Varner (18).

Deoxyribonuclease activity was determined both viscometrically and spectrophotometrically. The incubation medium was decanted into a 10-ml centrifuge tube along with 3 ml of a buffer rinse (0.1 mM sodium succinate [pH 5.2], 0.03 M $MgCl_2$). The tissue was then ground in a mortar with 5 ml of the same buffer, and both medium and extract fractions were centrifuged to remove wall material. The supernatant was used directly in the enzyme assay. Salmon sperm DNA (0.5 mg/ml) was dissolved in a buffer containing 0.03 M tris-HCl (pH 7.6), 0.15 M NaCl, and 0.03 M $MgCl_2$, and 5 ml was added to an Ostwald capillary viscometer, previously calibrated with buffer alone. The reaction was initiated by the addition of 20 μl of enzyme, and allowed to proceed with gentle shaking at 30 C for 30 min. Enzyme activity was expressed as the change in the reciprocal specific viscosity/min. For spectrophotometric assays, the substrate buffer was changed to 0.03 M tris-acetate (pH 6), as recommended by Brawerman and Chargaff (2) for the DNAse found in malt. An enzyme aliquot (80 μl) was added to 10 ml of double- or single-stranded DNA solution. Single-stranded DNA was prepared by heating the DNA solution for 10 min at 100 C, and rapidly cooling in an ice bath. This treatment resulted in a 33% increase in the absorbance at 260 nm. The reaction was carried out in a 25-ml Erlenmeyer flask at 30 C on a water bath shaker. One-ml fractions were removed every 5 min and added to tubes containing 1 ml of cold 0.02 M lanthanum nitrate, as described by Hanson and Fairley (10). After 40 min the tubes were centrifuged (10,000g, 20 min) and the supernatants were read at 260 nm.

RESULTS

Incubation of imbibed barley halfseeds in succinate buffer resulted in a 25 to 40% increase in the DNA content of the aleurone layer (Fig. 1A). The increase reached a plateau by 12 or 24 hr, and thereafter declined. The DNA content of GA-treated halfseeds did not increase as much as the controls, and declined more rapidly after 12 hr (Fig. 1A). γ-Irradiation (500 krads) effectively blocked the increase in the controls, but did not affect the decline in the hormone-treated tissue (Fig. 1A). γ-Irradiation does not interfere with GA-stimulated α-amylase production or release (data not shown). The DNA content of isolated aleurone layers decreased steadily during incubation,

and GA slightly accelerated the rate of DNA loss (Fig. 1B). Thus, halfseeds differ from isolated aleurone layers with respect to DNA metabolism.

Preliminary studies had shown that aleurone cells incorporated radioactive thymidine into DNA extracted by the Schmidt-Thannhauser-Schneider procedure (31, 32). Experiments were carried out to determine the effect of GA on the specific radioactivity of aleurone DNA. During the first 12 hr of treatment, GA increased the specific radioactivity of normal and γ-irradiated halfseed DNA by ~ 6% and ~ 16%, respectively (Table I). By 24 hr, the specific radioactivity of the hormone-treated DNA had declined significantly below the controls. However, GA also reduced the uptake of label (Table I), possibly due to isotope dilution (33). GA actually increased the specific radioactivity to uptake ratio at 24 hr by ~45% in the normal halfseeds and ~ 30% in the irradiated halfseeds (Table I). In isolated aleurone layers GA also reduced the specific radioactivity of DNA after 24 hr, but slightly increased (~ 18%) the specific radioactivity to uptake ratio (Table I).

Pulse-chase studies were performed in order to examine hormonal effects on DNA turnover under conditions in which a hormone-induced increase in the endogenous thymidine pool would be saturated by an exogenous supply of cold thymidine. Halfseeds were pulsed for 12 hr with radioactive thymidine, followed by a 12-hr chase in 10 mM cold thymidine plus or minus GA. As shown in Figure 2A, GA inhibited the decay of radioactivity from halfseed aleurone DNA. In the case of isolated aleurone layers, however, GA increased the rate of decay (Fig. 2B).

Attempts were made to characterize the labeled DNA on CsCl density gradients after purification by the procedure of Marmur (24). DNA purified by the standard Marmur procedure produced an asymmetrical peak on a CsCl density gradient (Fig. 3A). The shoulders on the light side of the peak corresponded to a white, flocculent precipitate. Figure 3B illustrates a similar preparation spun at a slower speed. Increased separation of the DNA peak from the precipitate has occurred, with radioactivity appearing in both peaks. Two observations suggest that the precipitate is a polysaccharide. First, it is anthrone-positive; and second, pretreatment of the DNA preparation with a mixture of α-amylase and invertase effectively eliminated the precipitate (Fig. 4A).

When aleurone cell DNA is pretreated with a mixture of α-amylase and invertase and spun on a CsCl density gradient, a single peak occurs at approximately 1.70 g/ml (Fig. 4A), similar to the previously reported values for barley DNA, *i.e.* 1.701

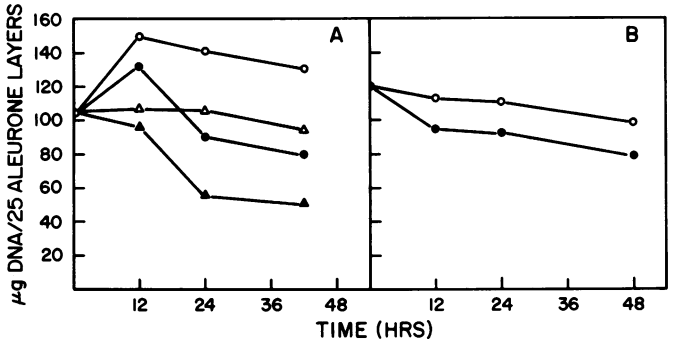

FIG. 1. Effect of GA on the DNA content of barley aleurone layers. Halfseeds were inbibed in 10 mM succinate buffer (pH 5.2) for 4 days prior to incubation. A: intact halfseeds incubated in buffer plus or minus GA (10 μM). Aleurone layers were isolated and rinsed thoroughly prior to extraction: (○——○), buffer control; (●——●), GA; (△——△), γ-buffer control; (▲——▲), γ-GA. B: isolated aleurone layers incubated in buffer plus or minus GA (10 μM): (○——○), buffer control; (●——●), GA. Data are an average of triplicate samples.

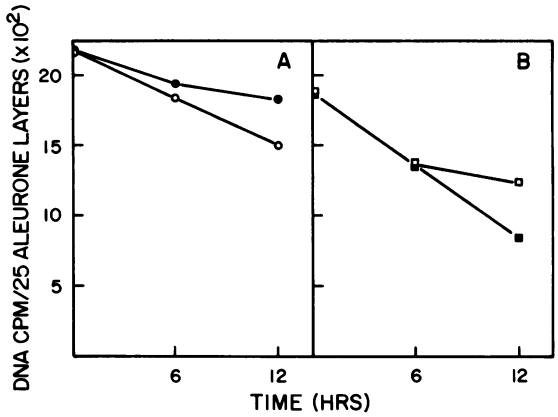

FIG. 2. Effect of GA (10 μM) on the lability of newly synthesized DNA in aleurone layers. Twenty-five halfseeds were imbibed for 3 days in 10 mM succinate buffer (pH 5.2) containing 4 μCi/ml [^{3}H-Me]thymidine (71.5 Ci/mmol). The halfseeds were then transferred to 25-ml flasks containing 5 ml of the same medium, and pulsed for an additional 12 hr on a shaker at 30 C. At the end of the pulse, the medium was decanted, the halfseeds were rinsed three times in sterile buffer and placed in flasks containing 10 ml of sterile buffer with 10 mM unlabeled thymidine. The tissue was shaken at 30 C during the chase period. A: halfseeds. Aleurone layers were isolated after the chase period, prior to extraction: (○——○), buffer control; (●——●), GA. B: isolated aleurone layers: (□——□), buffer control; (■——■), GA. Data are an average of triplicate samples.

Table I. Effect of GA and gamma irradiation on the incorporation of (^{3}H-Me) Thymidine into aleurone layer DNA

Ten halfseeds or isolated aleurone layers were incubated in 4 μCi/ml (^{3}H-Me)thymidine (71.5 Ci/mmol). The data are an average of triplicate samples.

Treatment	Uptake cpm		DNA cpm/mg DNA		DNA cpm/mg DNA / uptake cpm	
	12 hr	24 hr	12 hr	24 hr	12 hr	24 hr
Halfseeds						
-GA	446,410	241,610	21,833	17,362	0.0489	0.0719
+GA	413,740	106,450	23,173	11,111	0.0560	0.1044
-GA, gamma	336,670	185,160	29,884	32,125	0.0888	0.1738
+GA, gamma	326,800	113,790	34,872	25,652	0.1067	0.2254
Isol. Al. Layers						
-GA	----	528,300	----	34,210	----	0.0648
+GA	----	339,733	----	26,086	----	0.0768

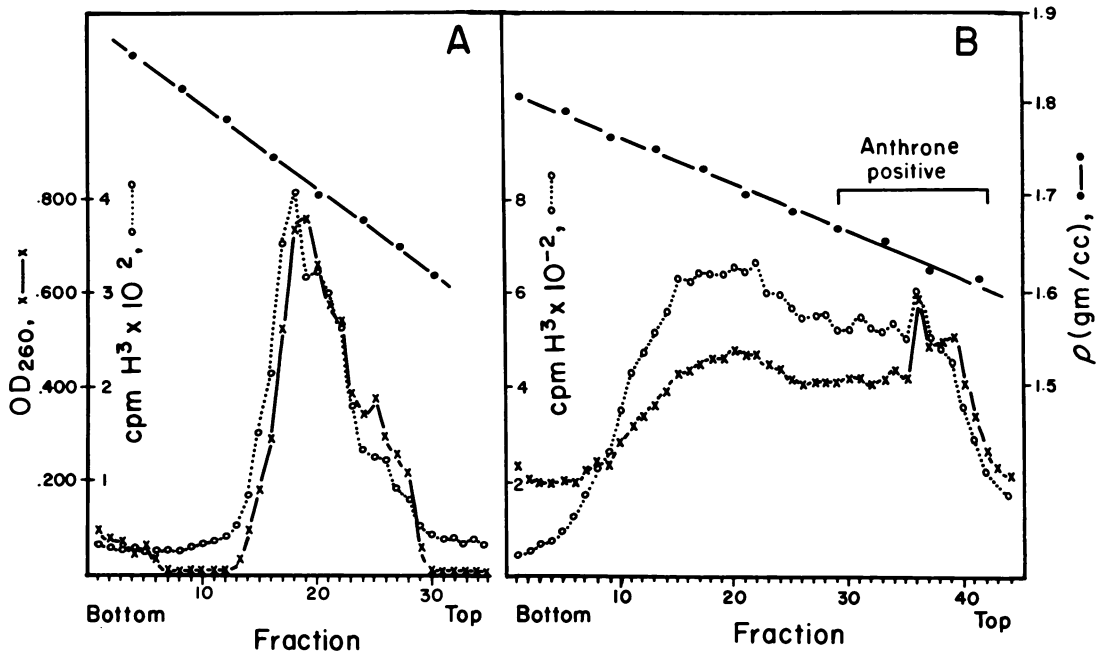

FIG. 3. CsCl density gradient profile of aleurone layer DNA prepared by Marmur procedure. Aleurone layers were incubated for 12 hr in 1 mM acetate-$CaCl_2$ buffer (pH 4.8) containing 4 μCi/ml [^{3}H-Me]thymidine (14.1 Ci/mmol). A: centrifuged for 42 hr, 39,000 k rpm; B: centrifuged for 40 hr, 29,000 k rpm.

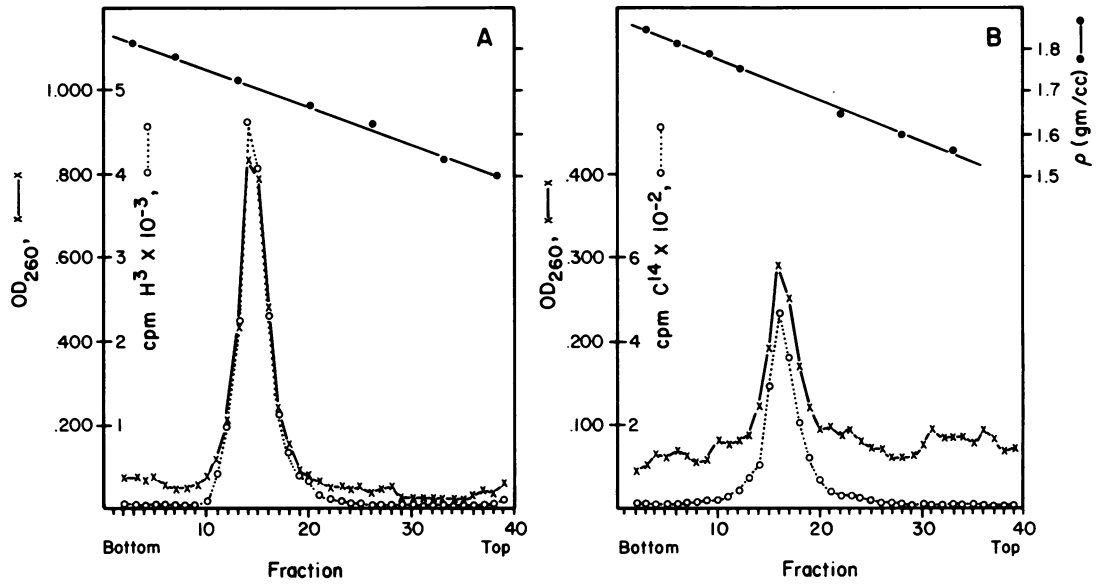

FIG. 4. CsCl density gradient profile of aleurone layer DNA treated with α-amylase and invertase. Centrifugation was at 39,000 k rpm for 44 hr. A: DNA labeled with [^{3}H-Me]thymidine; B: DNA labeled with [2-^{14}C]thymidine.

g/ml (1). In addition, the peak is completely eliminated by a treatment with DNAse (data not shown). The radioactivity exactly coincides with the absorbance peak. The same experiment performed with [2-^{14}C]thymidine also shows radioactivity exclusively associated with the DNA absorbance peak (Fig. 4B). The incorporation of ring-labeled thymidine indicates that the entire molecule is incorporated, and not just the methyl group. Hydrolysis and base separation by paper chromatography of the [^{3}H-Me]thymidine-labeled DNA confirmed that over 50% of the radioactivity was associated with thymine (data not shown).

If the alterations in nuclear DNA during aleurone cell development represent amplification of functionally specific genes, the possibility is introduced that the newly labeled DNA might differ in base composition from DNA extracted from whole seedlings. To test this possibility, [^{3}H-Me]thymidine-labeled DNA from GA-treated aleurone layers was centrifuged with a large amount of DNA from whole seedlings. The radioactive peak coincided exactly with the absorption peak, suggesting that the base composition of aleurone cell DNA does not significantly change during development (data not shown).

In order to confirm the presence of semiconservative replication in aleurone cells, density-labeling experiments were carried out with BUDR. Halfseeds were imbibed in the BUDR-containing medium of Haut and Taylor, including 0.1 mM aminopterin (11). Following imbibition, the halfseeds were transferred to the standard incubation medium, along with 4 μCi/ml [^{3}H]BUDR, for 16 hr. The buoyant density profile of the resulting aleurone cell DNA is shown in Figure 5A. A bimodal absorption curve is

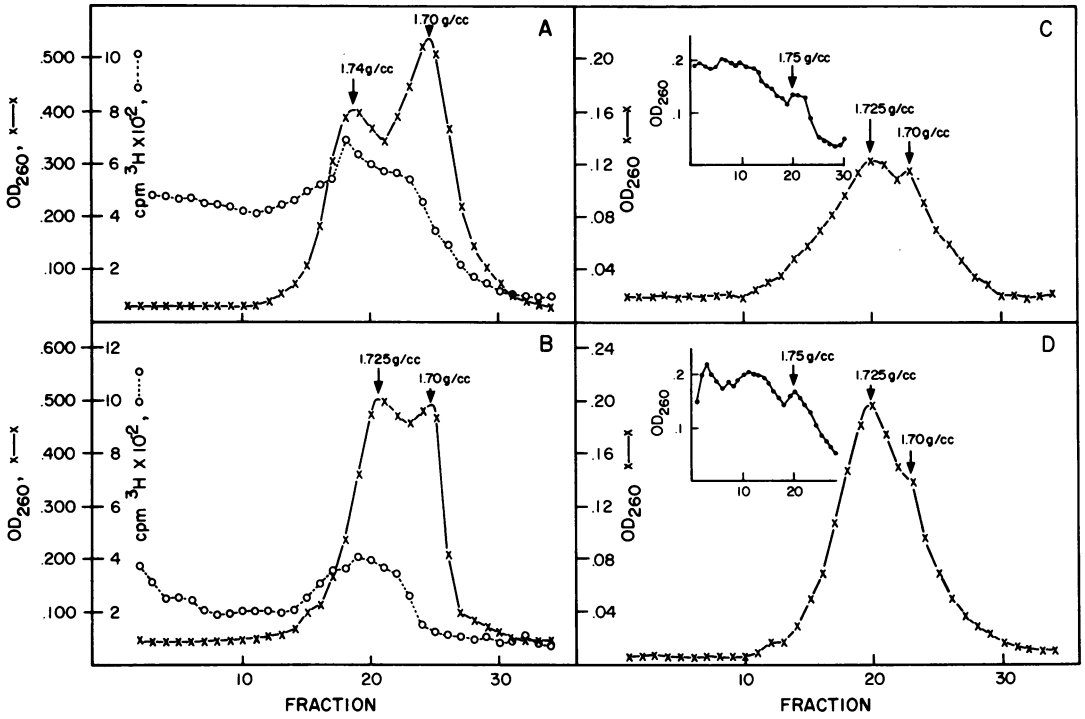

FIG. 5. Effect of GA on the buoyant density of halfseed aleurone layer DNA which has been density-labeled with BUDR. A: buffer control. Halfseeds were imbibed in unlabeled BUDR-containing medium, as described under "Materials and Methods," followed by incubation for 16 hr in 4 μCi/ml [³H]BUDR (12 Ci/mmol). B: GA (10 μM). Incubation as in A. C: buffer control. Halfseeds were imbibed and incubated in unlabeled BUDR medium for 24 hr. Inset shows the distribution of absorbance at 260 nm when fractions 13 to 23 were rerun in a CsCl gradient adjusted to pH 12 with NaOH. D: GA (10 μM). Treatment as in C. Approximate buoyant densities, based on refractive index measurements, are indicated for 96 hr, 32,000 rpm.

observed, with the radioactive peak roughly coinciding with the heavier absorption band. The buoyant density of the light band corresponds to unlabeled aleurone DNA (1.70 g/ml) while the heavy band corresponds to an almost fully substituted hybrid density-labeled species (1.74 g/ml). Density-labeled DNA from GA-treated halfseeds differs in several respects from the buffer control (Fig. 5B). The buoyant density of the heavy band is lower than the control (1.725 g/ml), and its specific radioactivity is also reduced, both of which can be accounted for by hormone-induced isotope dilution effects. However, the absorbance peak of the putative hybrid species is greatly increased, relative to the light band, by hormone treatment, suggesting a stimulation of semiconservative replication.

A similar experiment is shown in Figure 5, C and D. In this case, an attempt was made to saturate hormone-induced pool changes by administering 0.1 mM BUDR (unlabeled) throughout the incubation period. The length of the incubation was also extended to 24 hr. The resulting buoyant density profiles show that no difference was observed between the buoyant densities of the heavy bands in the hormone-treated and control DNA (1.725 g/ml). The reason for the reduced buoyant density of the heavy band in the control DNA is not clear, although the longer incubation period may be responsible. The significant point is that GA again enhanced the relative size of the heavy peak.

If this peak is, in fact, a hybrid density-labeled species, it should separate into two peaks when centrifuged under denaturing conditions (11). The pooled fractions from the heavy bands in Figure 5, C and D were therefore recentrifuged in alkaline CsCl (insets). As expected, a small peak occurred at about 1.73 to 1.75 g/ml, corresponding to unsubstituted single strands. However, instead of a single density-labeled peak, a broad band occurred from 1.75 g/ml to 1.90 g/ml. Such a broad band might be expected if: (*a*) extensive DNA repair had occurred; or (*b*) the chromosomes are replicating asynchronously, resulting in hybrid molecules with large variations in degrees of substitution. Since much of the DNA is located at 1.8 g/ml or lower, the buoyant density of fully substituted single strands in alkaline CsCl (11), the presence of some fully substituted single strands is inferred. Although there is some ambiguity in the alkaline profile, the results are consistent with the identification of the heavy peak as a hybrid species.

Density-labeling experiments with DNA from isolated aleurone layers resulted in a buoyant density profile resembling repair synthesis (27), *i.e.* no shift in the absorbance peak, but a slight shift in the radioactive peak (Fig. 6A). Similar results were obtained when ¹⁵N-labeled bases, in the presence of [³H]deoxyadenosine, were used in place of BUDR, in an attempt to amplify the density shift (Fig. 6B). Although a small density-labeled radioactive peak was observed, the single absorbance peak corresponded to unsubstituted aleurone DNA (1.70 g/ml), and most of the radioactivity was only slightly shifted toward the heavy side of the absorbance peak. It seems that the failure of isolated aleurone layers to accumulate DNA is due to a reduced capacity to carry out semiconservative replication.

We examined the possibility that GA alters DNA methylation, as it does RNA methylation (4). Table II summarizes the per cent incorporation of radioactivity from [³H-Me]methionine into the bases of aleurone cell DNA. Over 60% of the label is recovered in 5-methylcytosine, with no significant effect of the hormone on the percentage distribution. Although GA did not affect the pattern of base methylation, it substantially reduced the specific radioactivity of [³H-Me]methionine-labeled DNA (Table III). However, as in the case of [³H-Me]thymidine, precursor uptake was inhibited. When the data were expressed as a specific radioactivity to uptake ratio, the effect of the hormone on methylation appeared to be stimulatory (Table III).

Deoxyribonuclease has been shown to be present in malt (2), but there have been no reports of its production by isolated aleurone layers. We assayed for the enzyme using a viscometric procedure, in order to detect endonuclease activity. The time

course for the production and release of DNAse in response to GA is shown in Figure 7. A 16- to 18-hr lag period preceded the release of DNAse into the medium. GA exerted a strong control over the release of the enzyme, but increased only slightly its total activity (50%). When DNAse activity was measured spectrophotometrically, the preparation was about 2.5 times more active against single-stranded DNA than against double-stranded DNA, and it rapidly degraded poly(dAT), but was completely inactive against poly(dGC) (data not shown).

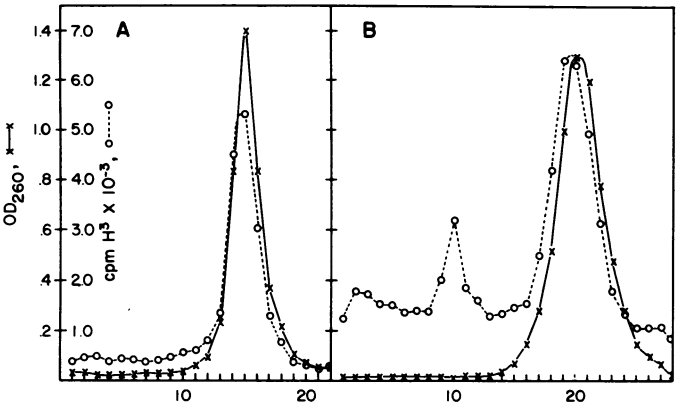

FIG. 6. Buoyant density profile of density-labeled DNA from isolated aleurone layers. A: BUDR. The tissue was treated as in Figure 5A, except that the halfseeds were stripped prior to incubation in [^{3}H]BUDR. The GA-treated aleurone DNA was similar and is not shown. The absorbance peak corresponds to unsubstituted DNA. B: ^{15}N. Halfseeds were imbibed in a mixture of ^{15}N-bases (10 mM) obtained from acid hydrolyzed *E. coli* DNA plus 5 μCi/ml [^{3}H]deoxyadenosine (6.4 Ci/mmol). Aleurone layers were then isolated and treated for an additional 24 hr in 10 ml of the same mixture. The GA-treated sample was similar and is not shown. The absorbance peak again corresponds to unsubstituted DNA.

Table II. Effect of Gibberellic Acid on the Percentage Distribution of Radioactivity from (^{3}H-Me) Methionine into the Bases of Aleurone Layer DNA

Isolated aleurone layers (100) were incubated in 20 ml succinate-$CaCl_2$ buffer (pH 5.2) containing 10 uCi/ml (^{3}H-Me)methionine (5 Ci/mmol) for 24 hr at 30 C. The DNA was isolated and processed as described under "Materials and Methods." The values represent the average ± SD of four separate experiments. Since the total cpm varied considerably from experiment to experiment, the data were expressed as per cent of the total cpm in the eight bases examined.

Base	Percent of Total	
	-GA	+GA
	cpm	
Adenine	11.3 ± 5.0	15.0 ± 2.5
Thymine	10.8 ± 4.0	7.1 ± 3.6
Cytosine	3.3 ± 3.0	3.5 ± 2.4
Guanine	4.4 ± 3.0	4.6 ± 1.7
5-Methylcytosine	63.9 ± 7.0	61.5 ± 8.5
6-Methyladenine	2.5 ± 4.0	2.1 ± 2.1
7-Methylguanine	4.75 ± 3 3	2.0 ± 1.4
5-Hydroxymethylcytosine	4.0 ± 4.0	3.85 ± 4.2

DISCUSSION

Recent cytophotometric studies have established that the nuclear DNA content of oat and barley aleurone layers is not constant during development (20, 23). In barley, cytodifferentiation is accompanied by a gradual increase in nuclear DNA content, from 3 to 6C to 9 to 12C. This increase in nuclear DNA resumes when dry halfseeds have been fully imbibed on water for 3 days (20).

Since it is not possible to separate sufficient quantities of immature aleurone layers from the starchy endosperm for biochemical analysis, we have examined the capacity of mature, fully imbibed aleurone layers to synthesize DNA. Using biochemical extraction methods we have confirmed that aleurone nuclei continue to synthesize DNA during incubation of the halfseeds. The over-all effect of GA is to reduce net DNA accumulation in the tissue. However, during the first 12 hr of treatment, at least, hormone-induced reduction in total DNA is accompanied by a slight increase in the specific radioactivity of the DNA in labeling experiments, suggesting DNA turnover. The reduction in specific radioactivity at 24 hr may be a consequence of hormonal inhibition of uptake due to increases in endogenous pools, as in the case of amino acids (33). Thus, the

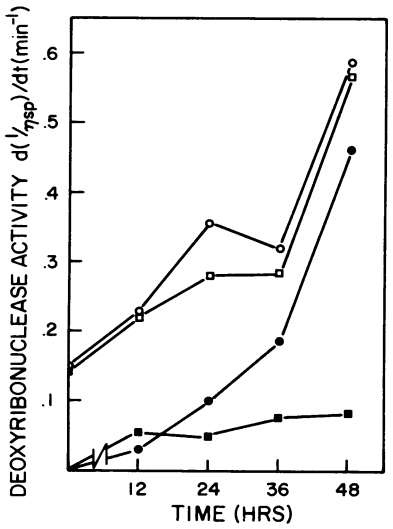

FIG. 7. Time course of deoxyribonuclease activity. Ten aleurone layers were incubated with 10 μM GA: (■——■), buffer control medium; (□——□), buffer control-extract; (●——●), GA-medium; (○——○), GA-extract.

Table III. Effect of gibberellic acid on the specific radioactivity of (^{3}H-Me) Methionine-labeled DNA extracted from halfseed aleurone layers

Twenty-five halfseeds were incubated in 5 ml of succinate-$CaCl_2$ buffer (pH 5.2) containing 4 μCi/ml (^{3}H-Me) methionine (5 Ci/mmol) at 30 C. After incubation, the aleurone layers were isolated at 4 C, rinsed repeatedly with ice water, frozen in liquid N_2, and lyophilized. DNA determinations were performed after extracting nucleic acids by the method of Laulhere and Rozier (21). DNA cpm were extracted from the nucleic acids by the Schmidt-Thannhauser-Schneider procedure (34). The data are an average ± standard deviation of triplicate samples.

Treatment	Uptake cpm		DNA cpm/mg DNA		DNA cpm/mg DNA / Uptake cpm	
	12 hr	24 hr	12 hr	24 hr	12 hr	24 hr
-GA	759,450	728,700	84,250	54,017	0.1109	0.0741
+GA	458,950	430,100	48,576	42,361	0.1058	0.0985

specific radioactivity to uptake ratio may more accurately reflect the stimulatory effect of GA on DNA synthesis. GA has recently been shown to stimulate DNA synthesis in cucumber hypocotyls (19).

The total DNA content of isolated aleurone layers does not increase during incubation, although thymidine is incorporated into DNA. Gibberellic acid augments only slightly the specific radioactivity to uptake ratio (18%) in isolated aleurone layers, compared to 45% in halfseeds. In pulse-chase experiments, it was found that GA accelerated the loss of label from isolated aleurone DNA, but reduced it in halfseeds. The reduction in halfseeds can be rationalized on the basis of more rapid reutilization of isotope due to hormone-induced synthesis. Density-labeling experiments with BUDR demonstrated that: (*a*) DNA synthesis in the aleurone cells of halfseeds is by semiconservative replication; and (*b*) in halfseeds GA stimulates semiconservative replication. The density-labeling data thus support the incorporation studies.

In addition to semiconservative replication, aleurone cells also possess a repair mechanism which can be activated by γ-irradiation. Isolated aleurone layers incorporated [^{3}H-Me]thymidine almost exclusively by DNA repair. Either the starchy endosperm provides factors necessary for DNA synthesis, or the procedure for isolating aleurone layers, which possibly involves some tissue damage, induces a wound response inhibitory to semiconservative replication. Since GA increases the specific activity to uptake ratio in both γ-irradiated halfseeds and isolated aleurone layers, the hormone apparently stimulates repair synthesis, although not to the same degree as semiconservative replication. The function of repair synthesis in the intact tissue is unknown. It may serve to protect active genes from premature destruction by nucleases. Since we were not aware of a means to inhibit repair synthesis specifically, we were unable to determine what role it plays in the hormone response.

DNA methylation has recently received attention in the literature because of its relation to restriction endonucleases. Microbial restriction endonucleases recognize specific sequences in DNA, and may be blocked by methylated bases, usually 6-methyladenine (25). Sager (28) speculated that *Hordeum vulgare* might contain modification and restriction enzymes since it was able to recognize and degrade chromosomes of *Hordeum bulbosum* during hybrid formation, resulting in a haploid embryo with only *H. vulgare* chromosomes. Subrahmanyan *et al.* (30) found that chromosomes of *H. vulgare* and *H. bulbosum* were both cleaved by a mixture of restriction enzymes from *Haemophilus influenzae*. If similar restriction endonucleases were present in aleurone cells, GA could conceivably stimulate DNA degradation by reducing DNA methylation. Although it was found that GA reduced the specific radioactivity of [^{3}H-Me]methionine-labeled DNA, this reduction is believed to be due to a reduction in uptake. GA did not reduce the per cent incorporation into 5-methylcytosine, the major methylated base.

The deoxyribonuclease assayed in this study was present in the tissue prior to treatment with GA, and its activity was enhanced only slightly by the hormone. The kinetics of the release of the enzyme suggests some similarities to ribonuclease (5). Wilson (36) has pointed out that no specific deoxyribonucleases have yet been found in plants. The enzyme preparation is more active against single-stranded DNA, and prefers poly(dAT) sequences to poly(dGC) sequences. These characteristics suggest that deoxyribonuclease activity, as well as some ribonuclease activity, is due to the nonspecific nuclease I. Previous reports of deoxyribonuclease activity in wheat embryos (10) and in barley malt (2) are both attributed by Wilson to nuclease I (36). Leshem *et al.* (22) have recently reported that GA induces a partial opening of double-stranded DNA in *Zea mays*. It is possible that GA-enhanced DNA degradation in aleurone layers could be related to a similar phenomenon.

In the absence of a demonstrated physiological role for DNA turnover in aleurone layers, the functions of storage and mobilization inevitably come to mind. A parallel situation has been suggested for pea cotyledons (3, 29). It has been proposed that much of the DNA and RNA in these cells are nonfunctional storage forms of nucleotides which are broken down for transport to the embryonic axis during germination (26, 29). Much of the DNA and RNA of aleurone cells may also be nonfunctional storage products. Indeed, it is possible to reduce RNA synthesis greatly with 5-fluorouracil without affecting the GA-stimulated α-amylase production (37). If the phases of DNA storage and degradation overlap during germination, the result is a period of DNA turnover. GA may act as a governor, hastening the onset of the mobilization phase.

Acknowledgments – We are grateful to R. L. Jones and F. Wilt for their helpful suggestions and encouragement during the early part of this work. We also wish to thank our colleagues, J. Feldman and P. Dice, for their valuable comments and criticisms.

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