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A Correlation between a Ribonucleic Acid Fraction Selectively Labeled in the Presence of Gibberellic Acid and Amylase Synthesis in Barley Aleurone Layers

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ABSTRACT

The effects of gibberellic acid on the incorporation of radioactive uridine and adenosine into RNA of barley aleurone layers were investigated using a double labeling method combined with acrylamide gel electrophoresis. After 16 hours of incubation, gibberellic acid stimulated the incorporation of label into all species of RNA, but the effects were very small (0–10%) for ribosomal and transfer RNA and comparatively large (up to 300%) for RNA sedimenting between 5S and 14S. This result was obtained for both isolated aleurone layers and for layers still attached to the endosperm. A similar but less marked pattern occurred in layers incubated for 8 hours, but the effect was not observed after 4 hours. The gibberellic acid-enhanced RNA labeling was not due to micro-organisms. The following evidence was obtained for an association between the gibberellic acid-enhanced RNA synthesis and α-amylase synthesis: (*a*) synthesis of α-amylase took place in parallel with incorporation of label into gibberellic acid-RNA; (*b*) actinomycin D inhibited amylase synthesis and gibberellic acid-RNA by similar percentages; (*c*) 5-fluorouracil halved incorporation of label into ribosomal RNA but had no effect on amylase synthesis and gibberellic acid-RNA; and (*d*) abscisic acid had little effect on synthesis of RNA in the absence of gibberellic acid, but when it was included with gibberellic acid the synthesis of both enzyme and gibberellic acid-RNA was eliminated. We conclude that large changes in the synthesis of the major RNA species are not necessary for α-amylase synthesis to occur but that α-amylase synthesis does not occur without the production of gibberrellic acid-RNA. Gibberellic acid-RNA is probably less than 1% of the total tissue RNA, is polydisperse on acrylamide gels, and could be messenger species for α-amylase and other hydrolytic enzymes whose synthesis is under gibberellic acid control.

The induction of α-amylase synthesis in endosperm halves of barley seeds by GA was discovered by Paleg (14) and by Yomo (22). Later it was found (1, 13, 15, 20) that the site of production was the aleurone layer and that the GA effect could be obtained in aleurone layers isolated from the starchy endosperm. Filner and Varner (7) showed that α-amylase was synthesized *de novo*, and the inhibitor studies of Varner *et al.* (21) indicated the dependence of enzyme synthesis on RNA synthesis. Chandra and Varner (3) studied the incorporation of radioactive precursors into RNA and observed a 50 to 70% stimulation of incorporation into salt-soluble RNA by GA over the first 24 hr of incubation. Chandra and Duynstee (2) fractionated aleurone RNA on methylated albumin kiesulguhr columns and showed that in the presence of GA a specific fraction of RNA appeared as a shoulder on the heavy ribosomal peak.

The present work was undertaken to examine further the implied association between RNA synthesis and enzyme synthesis. Acrylamide gel electrophoresis was used to fractionate extracted RNA so that the fractions could be distinguished by their molecular weights. A double labeling method was employed to maximize the chances of detecting GA-induced changes in RNA. A preliminary account of this work has been published (23).

MATERIALS AND METHODS

Tissue Preparation and Incubation. Barley seed (*Hordeum vulgare* L. cv. Himalaya) was used as the experimental material. Seed imbibition, isolation of the aleurone layers, and the amylase assays were carried out as described by Chrispeels and Varner (4). All amylase measurements are given in units; one unit is the amount of amylase necessary to give 1 unit per min change in A_{620}.

In some experiments (indicated in the text) de-embryonated seeds, called half seeds, were used. The incubation medium contained 10 mM calcium chloride, 1 mM sodium acetate buffer, pH 4.8, and 1 drop of chloramphenicol solution (0.5 mg/ml) in a final volume of 2 ml. GA at 1 μM and labeled adenosine and uridine were added when appropriate. In some experiments inhibitors were also present in the incubation medium.

Double Labeling. To each of two 30-ml Erlenmeyer flasks (20 layers/flask), 20 μc of ^{3}H-adenosine (2 c/mmole, Radiochemical Centre) and 20 μc of ^{3}H-uridine (0.5–3 c/mmole) were added, and to a third flask (20 layers) 5 μc of ^{14}C-adenosine (450 mc/mmole) and 5 μc of ^{14}C-uridine (405 mc/mmole) were added.

Although carrier nucleoside was not added in these experiments, incorporation into the ribosomal species was linear over the 16-hour period. It is unlikely therefore that dilution of label by endogenous pools contributed to the results we observed.

The flasks were incubated at 25 C for the required time in a shaking water bath in the dark and then the layers were removed and washed with 3 × 3-ml aliquots of 1 mM uridine and

adenosine. The 40 layers incubated with ^3H were then mixed with the 20 incubated with ^{14}C and RNA was prepared from them. This treatment was designated −GA/−GA as neither the ^3H- nor the ^{14}C-labeled layers had been exposed to GA.

Another set of labeled layers was prepared, identical to the first in every respect except that the ^3H-labeled medium contained 1 μM GA. After mixing the layers this treatment was called +GA/−GA as the ^3H-labeled layers had been exposed to GA but the ^{14}C-labeled ones had not.

Nucleic acids were prepared by a modification of the method of Ingle and Burns (8), by a modification of the method of Solymosy *et al.* (19) or by a modification of the method of Click and Hackett (5).

In the first case the layers were immersed in phenol and buffer, chopped for 1 min in a VirTis homogenizer run at full speed and then stirred for 4 min at low speed. The method of Ingle and Burns (8) was then followed to the ethanol precipitation step. In the second case the layers were ground for 1 min in the VirTis homogenizer in 20 ml of 50 mM tris-HCl buffer, pH 7.6, containing 1% sodium dodecyl sulfate and 5 mM $MgCl_2$ and 0.6 ml of DEP[1] ("Baycovin," Bayer Ltd., Leverkusen, Germany). The homogenate was incubated at 37 C for 5 min, then centrifuged at 12,000*g* for 10 min. Sodium chloride (2 g) was dissolved in the supernatant, and the fluid was incubated again for 5 min at 37 C and centrifuged at 12,000*g* for 10 min. The supernatant was poured into 50 ml of cold absolute ethanol and kept overnight at −10 C. In the third case 0.2 ml of DEP were added to the buffer phenol mixture of Click and Hackett and the tissue was ground for 1 min in the VirTis homogenizer. Otherwise the procedure of Click and Hackett (5) was followed.

Total uptake of uridine and adenosine was measured by taking a 50-μl aliquot from the initial homogenate, counting it for ^3H and ^{14}C and calculating the ratio of ^3H dpm to ^{14}C dpm. The −GA/−GA gave the standard ratio, and comparison of this with the +GA/−GA ratio showed whether GA had affected uptake.

In all three methods the precipitated RNA was recovered by centrifugation and was dissolved in 3 ml of 0.5 M sodium acetate, pH 5.8, equilibrated with DEP. After short term incubations of aleurone tissue difficulty was experienced in redissolving the RNA prepared by the two phenol methods and the DEP method was preferred in these cases. The RNA was precipitated with 0.4 ml of 1% cetyltrimethylammonium bromide, washed with 70% ethanol containing 0.1 M sodium acetate, according to the method of Ralph and Bellamy (18), and dissolved in electrophoresis buffer (12).

The three methods of RNA preparation gave the same results. Variations in the magnitude of the effects to be described were observed on different occasions but these variations could not be associated with the use of any particular preparation method.

Thirty to 40 μg of RNA were subjected to electrophoresis on 7-mm diameter 2.4% acrylamide gels according to the method of Loening (12) but modified by the addition of 0.5% agarose (6). The gels were scanned at 265 nm in a Joyce-Loebl Chromoscan or at 260 nm in a Gilford 240 spectrophotometer with a linear transport attachment. The gels were then cut into 1 mm slices; each slice was transferred to a scintillation vial, 0.5 ml of NCS solubilizer (Amersham/Searle) was added, and the vials were heated for 2 hr at 60 C. Eight milliliters of scintillation fluid (6 g of PPO per liter of toluene) were added and, after standing overnight in darkness, the vials were counted in the ^3H and ^{14}C channels of a Beckman CPM 100 counter equipped with an external standard channel. The dpm values for both ^3H and ^{14}C were calculated from efficiency and "spillover" curves obtained with the aid of internal standards. The ratios of ^3H dpm to ^{14}C dpm were calculated.

When GA was absent from both the ^3H and ^{14}C treatments the ^3H dpm to ^{14}C dpm ratios would be expected to be constant over the length of the gel and deviation from constancy would be a measure of the variability of the method. If the synthesis of any particular species of RNA were promoted by GA, this species would contain more ^3H label than others and consequently the ^3H dpm to ^{14}C dpm ratios would be raised in the region of the gel in which it was localized. Thus a rise in the observed ratios in any particular region of a gel indicates stimulation by GA of incorporation into the species of RNA which electrophoreses to that region.

Actinomycin D and 5FU were purchased from the Sigma Chemical Company, and ABA was a gift from Hoffman La Roche.

RESULTS

Time Course of α-Amylase Production in Isolated Aleurone Layers and Half Seeds. Table I shows total α-amylase production for isolated layers and for half seeds at 0, 4, 8, and 16 hr which were the times used in the labeling experiments. In the first 4 hr little synthesis took place in response to GA. At 8 hr amylase synthesis had begun and a large increase took place between 8 and 16 hr.

Effects of GA on Label Incorporation into RNA of Aleurone Layers. In preliminary experiments the effect of GA on total incorporation into RNA of isolated layers was measured by comparisons of incorporation of ^3H-uridine into the cold trichloroacetic acid insoluble fractions prepared from layers grown in the presence and absence of GA. We were unable to establish any effect of GA on this incorporation. It seemed unlikely therefore that GA had any effect on incorporation into the major RNA species. However, it was possible that there were effects on incorporation into minor species which could be discerned only when the RNA was isolated and fractionated. Acrylamide gel electrophoresis was chosen as the fractionation method because it separates the RNA species according to their molecular weights. In Figure 3 (−GA/−GA) the distribution of counts obtained from RNA prepared from isolated layers incubated for 16 hr in the presence of ^3H-labeled uridine and adenosine is shown. The regions of high count correspond to the ribosomal and transfer RNA in which, from the results with the trichloroacetic acid precipitates, no GA promoted differences seemed probable. If differences in minor species present in regions of low counts were to be detected, a very sensitive method was clearly necessary.

Table I. *α-Amylase Produced by 10 Isolated Aleurone Layers or Half Seeds with and without GA*

Time of Incubation	Isolated Layers		Half Seeds	
	−GA	+1 μM GA	−GA	+ 1 μM GA
hr	*units*			
0	4.8	4.8	4.8	4.8
4	3.6	5.0	6.8	8.3
8	7.7	15.2	6.5	10.4
16	4.8	104.0	8.9	50.8

[1] Abbreviations: DEP: diethyl pyrocarbonate; Act D: Actinomycin D; 5FU: 5-fluorouracil; GA-RNA: gibberellic acid-enhanced RNA synthesis.

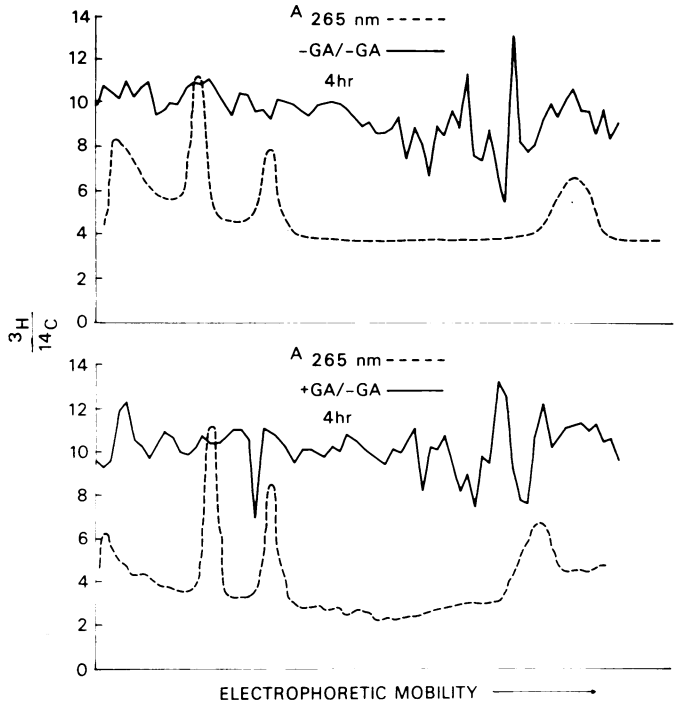

FIG. 1. A_{265nm} traces of RNA fractionated on acrylamide gels and $^3H/^{14}C$ dpm ratios of labeled RNA contained in the gel slices. The RNA had been prepared from isolated aleurone layers incubated for 4 hr. In −GA/−GA neither the ^3H nor the ^{14}C-labeled layers received GA, in +GA/−GA only the ^3H-labeled layers received GA.

For this reason the double labeling method used by Patterson and Trewavas (16) for proteins and by Chandra and Duynstee (2) for RNA was adopted.

Figure 1 shows the absorbance ($A_{265\ nm}$) traces of fractionated RNA and the ^3H dpm to ^{14}C dpm ratios obtained when isolated layers were incubated for 4 hr with or without GA and the RNA had been prepared by the DEP method. The nucleic acid peaks from left to right correspond to DNA, 25S RNA, 18S RNA, and 4S RNA. The plots of the ratios for the +GA/−GA and −GA/−GA treatments were indistinguishable and there was no change in the average ratio. Therefore there was no evidence of a GA effect on incorporation into RNA during the first 4 hr of incubation.

After 8 hr incubation (Fig. 2), the overall +GA/−GA ^3H to ^{14}C ratio was higher than for the −GA/−GA treatment and this effect was greatest in the 5S to 14S region of the gel where it rose to about 12 compared with a value of about 8 in the −GA/−GA treatment. Thus in this time GA had stimulated incorporation into all species of RNA but most strongly into those which sediment between 5S and 14S.

Figure 3 shows the effect of 16 hr of incubation in the presence and absence of GA. Again the +GA/−GA ^3H to ^{14}C ratio was higher over the entire scan though only minimally so in the ribosomal and transfer RNA regions. In the 5S to 14S region there was a very marked increase in ratio and the incorporation in this region was stimulated 3-fold by GA. There was also a ratio rise between the ribosomal peaks and a sharp increase below the 4S region. These characteristics are seen in the other 16-hr incubations (Figs. 4, 5B, 6B, 7B) although there was variation in their magnitude on different occasions. The uptake ratios (not available for Fig. 4) showed that these results could not be accounted for by any effects of GA on label uptake.

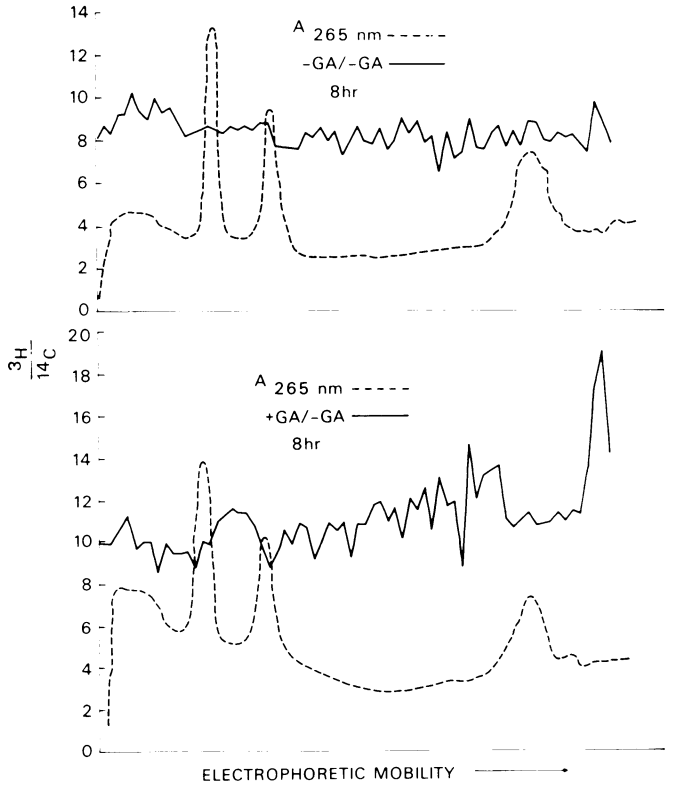

FIG. 2. As in Figure 1 except that the RNA had been prepared from isolated aleurone layers incubated for 8 hr.

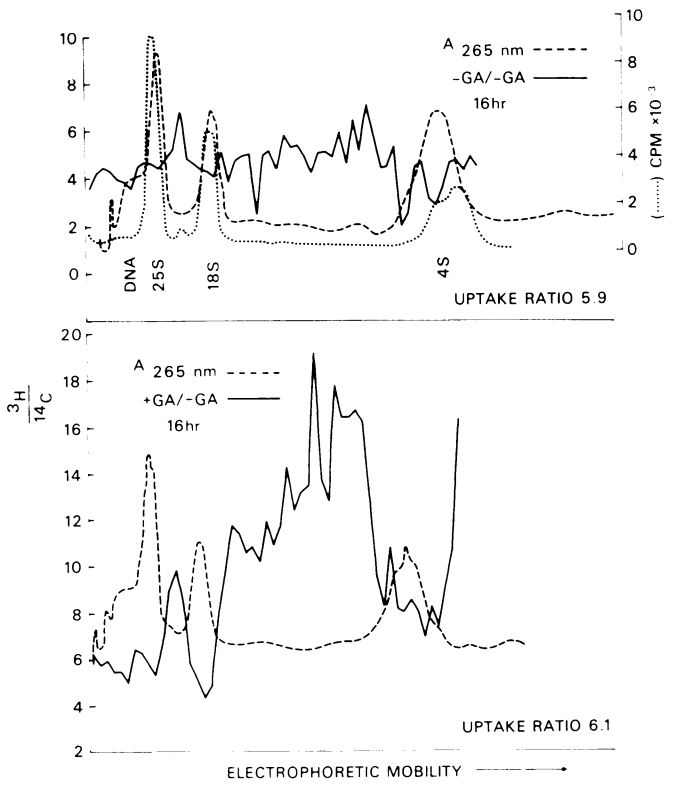

FIG. 3. As in Figure 1 except that the RNA had been prepared from isolated aleurone layers incubated for 16 hr.

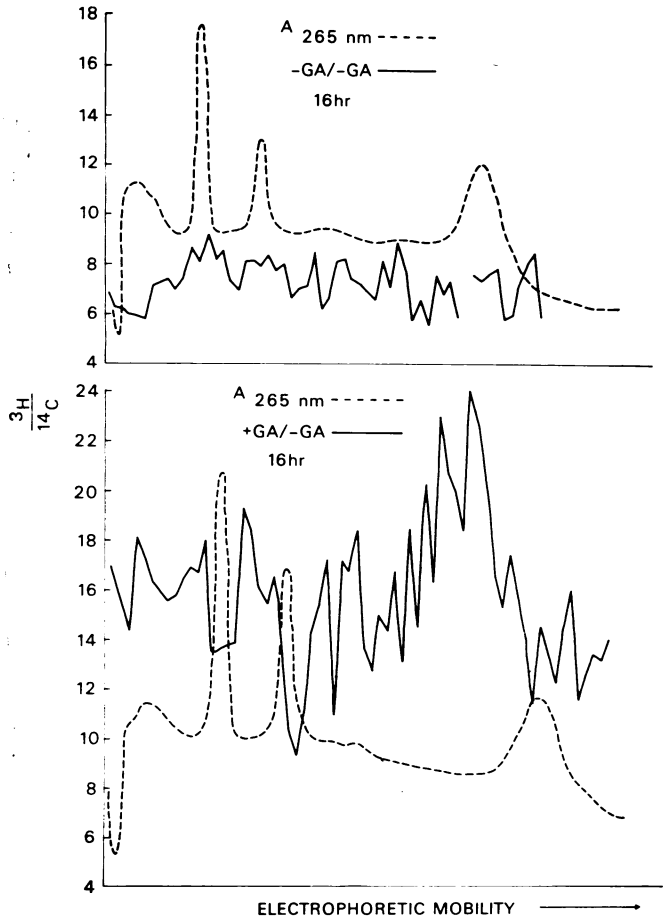

FIG. 4. As in Figure 1 except that the RNA had been prepared from aleurone layers attached to endosperm which had been incubated for 16 hr.

Figure 4 shows that the same effect occurred when half seeds were incubated in the presence of GA for 16 hr and the aleurone layers were isolated at the end of the incubation. As in the first case more uridine and adenosine were incorporated into all species of RNA in the presence of GA and there was a 3-fold increase of label incorporated in the 5S to 14S region. Therefore the stimulation of incorporation into polydisperse RNA occurred also in layers attached to the endosperm.

The possibility exists that the GA effect could have arisen through stimulation of growth of micro-organisms by GA. To account for the effect, large numbers of micro-organisms would have had to adhere to the washed isolated layers and we assumed that if this happened the medium also would contain many micro-organisms. We therefore measured the incorporation of labeled uridine and adenosine into cold trichloroacetic acid precipitable material from the incubation medium. There was incorporation into this fraction in the −GA treatment and this was doubled when GA was present. However if, before the addition of TCA, the medium was spun at 20,000*g* or was passed through a bacterial filter the TCA precipitates prepared from the supernatant or filtrate contained the same number of counts as the uncentrifuged or unfiltered material. We concluded therefore that the observed incorporation into medium material had been mediated by the aleurone tissue and not by micro-organisms. Hence GA-RNA is a consequence of the metabolic activity of aleurone layers and not of that of micro-organisms.

Effects of Act D, 5FU and ABA on RNA and Enzyme Synthesis in Aleurone Layers Incubated for 16 hr. Act D is a well known inhibitor of RNA synthesis and is known to prevent α-amylase synthesis (4). Its effect on enzyme production at a concentration of 100 μg/ml is shown in Table II. The treatment decreased the amount of enzyme by about 90%.

Figure 5, A and B, shows the effect of 100 μg/ml of Act D on RNA synthesis as measured by a double labeling experiment. In A the ^3H and ^{14}C ratios given by the −GA/−GA and (−GA + Act D)/−GA treatments are compared, and in B those from the +GA/−GA and (+GA + Act D)/−GA treatments. From A it is seen that the mean value of the ^3H to ^{14}C ratio line for the −GA/−GA treatment is about 6.5 and that the ratios for the (−GA + Act D)/−GA treatment do not anywhere reach this figure. In the ribosomal regions the ratios are less than three hence incorporation into these species of RNA was reduced to half by Act D. In the 5S to 14S region incorporation was only slightly reduced. This behavior is in agreement with the results of others (17) which show that synthesis of ribosomal RNA is more sensitive to Act D than is that of other species of RNA.

Figure 5B shows the effect of the inclusion of Act D with GA. Again incorporation into the ribosomal peaks was reduced to less than half of that in the +GA/−GA control. Integration between the ratio lines in the 5S to 14S region showed that Act D had reduced incorporation by about 75% in this region. The uptake ratio figures showed that changes in uptake could not account for these results.

Table II also shows the effect of 5FU on enzyme synthesis. Even at the high concentration of 1 mM, total enzyme production was unaffected. In Figure 6, A and B, the effects of 5FU on incorporation into RNA are shown. In A the ^3H to ^{14}C ratios given by the −GA/−GA and (−GA + FU)/−GA treatments are compared and in B those from the +GA/−GA and (+GA + FU)/−GA treatments. From A it is seen that FU halved incorporation into the ribosomal peaks but had little effect on incorporation in the 5S to 14S region. This result is in general agreement with the data of Key and Ingle (11). From B it is seen that the stimulation by GA of the ^3H to ^{14}C ratios in the 5S to 14S region of the +GA/−GA treatment is in this case less than usual. This variability has occasionally been encountered but we do not know why it occurs. The 5S to 14S peak resulting from the (+GA + FU)/−GA treatment was not reduced and was even larger than the con-

Table II. *Production of α-Amylase by 10 Isolated Aleurone Layers in the Presence and Absence of Act D, 5FU, and ABA after 16 hr Incubation*

Treatment	Activity of α-Amylase
	units
No GA	9.2
GA 1 μM	106.8
GA 1 μM + Act D 100 μg/ml	10.9
No GA	7.0
No GA + 5FU 1 mM	4.4
GA 1 mM	116.0
GA 1 mM + 5FU 1 mM	115.6
No GA	8.4
No GA + ABA 5 μM	1.4
GA 1 μM	86.3
GA 1 μM + ABA 5 μM	6.7

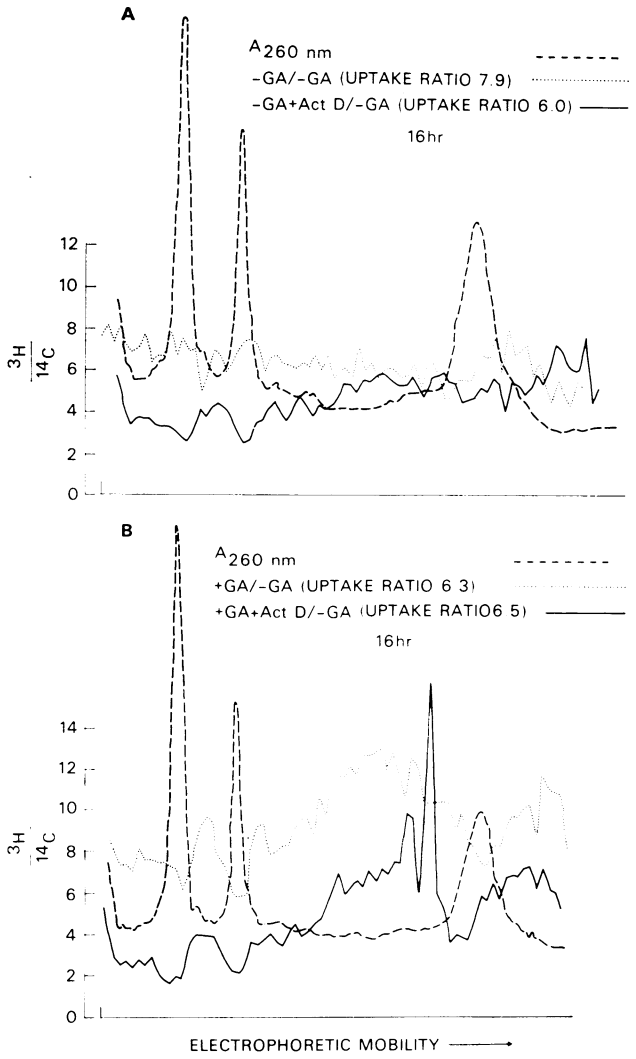

FIG. 5. A_{260nm} traces of RNA fractionated on acrylamide gels and $^3H/^{14}C$ dpm ratios of labeled RNA contained in the gel slices. The RNA had been prepared from isolated aleurone layers incubated for 16 hr. A: In −GA/−GA neither the ^3H nor the ^{14}C-labeled layers received GA, in (−GA + Act D)/−GA the ^3H-labeled layers received Act D and GA was not included with either label. B: In +GA/−GA only the ^3H-labeled layers received GA and in (+GA + Act D)/−GA the ^3H-labeled layers received both GA and Act D and the ^{14}C-labeled layers received neither substance.

trol +GA/−GA, but this was probably a result of the smaller than usual stimulation of the control.

ABA at 5 μM completely inhibited enzyme synthesis (Table II). Its effect at the same concentration on incorporation is shown in Figure 7. In A the ^3H to ^{14}C ratios given by the −GA/−GA and (−GA + ABA)/−GA treatments are compared and in B those from the +GA/−GA and (+GA + ABA)/−GA treatments. It is seen from A that ABA inhibited incorporation into all species of RNA to a small extent and that there were no differential effects. This was not accounted for by a change in uptake. From B it is clear that when GA was present ABA abolished all the differential stimulations and lowered the ratio almost to the (−GA + ABA)/−GA level shown in A.

DISCUSSION

We have found that GA stimulates incorporation of uridine and adenosine into RNA and hence we assume that GA stimulates RNA synthesis, but the effect is not the same for all RNA species. In many experiments incorporation of label into ribosomal RNA and 4S RNA was stimulated by GA, but only to a very small extent, and in some cases there was no effect at all on the major RNA species. Since almost all of the labeled nucleoside incorporated goes into the major RNA species (Fig. 3), our results are apparently at variance with those of Varner *et al.* (21), who found that GA significantly stimulated incorporation of uridine, adenine and ^{32}P into total RNA. Their data indicate that there is a general increase in rate of the synthesis of the major species. As they added GA and label to dry de-embryonated seed (aleurone layer plus endosperm), it is possible that the differences between their results and ours arise either because they were measuring GA induced changes associated with imbibition or GA induced changes dependent on the presence of endosperm. We attempted to avoid such complications by isolating the aleurone layers from seeds which had been allowed to imbibe water for 3 days in the absence of GA. However, in the experiment (Fig. 4) in which GA was given to imbibed de-embryonated seeds, we did observe a promotion of label incorporation into the major RNA species. Therefore it seems likely that the apparent conflict between our results and those of Varner *et al.* (21) can be explained by the fact that in their experiments GA was given in the presence of endosperm whereas in most of ours endosperm was not present when GA was given. Additional evidence that synthesis of major species of RNA is not an essential part of amylase induction by GA is that 5FU inhibited

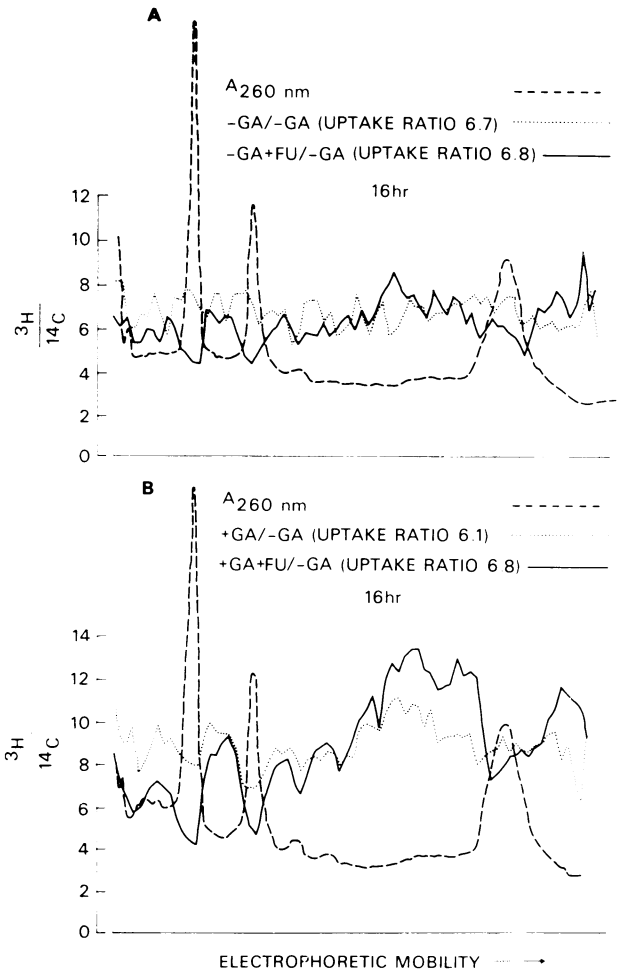

FIG. 6. As in Figure 5 with 5FU replacing Act D.

ribosomal and 4S RNA synthesis by about 50% while not affecting α-amylase synthesis.

The major changes in RNA following GA treatment occurred in species migrating on acrylamide gels between the ribosomal RNA peaks, between the light ribosomal and the 4S RNA (the 5 to 14S region) and frequently below the 4S RNA. In the following discussion we shall refer to the RNA which gives rise to these high ratios as GA-RNA.

It will be noticed that the high ratios occur where total counts are low so that the large GA-induced changes appear to involve only a very small fraction, possibly less than 1% of the total RNA of the cell. However, it must be pointed out that we cannot assume that GA-RNA occurs only on those portions of the gel where we observe the high ratios. The numbers of counts associated with the highest ratios are small compared to those associated with the ribosomal peaks (about 300 ^3H cpm compared with 10 to 15,000 ^3H cpm per gel fraction) so that counts incorporated into GA-RNA which are sufficient to cause substantial ratio changes in regions of low counts would not produce comparable changes in the ribosomal regions because of the overwhelming effect of the counts in ribosomal or 4S RNA. Hence it may be that GA-RNA occurs over most or all of the gel, not only in those regions in which we observe it. These strictures do not cast doubt upon the reality of the ratio increases we observe but they do indicate that we may be seeing only part of the full GA effect on RNA synthesis.

In this study we have presented evidence to demonstrate a correlation between GA-RNA synthesis and α-amylase synthesis. The increases in GA-RNA and α-amylase syntheses are simultaneous. Act D which inhibited enzyme production by 90% gave a comparable inhibition of 5 to 14S GA-RNA while major species of RNA were less affected. 5FU inhibited ribosomal and 4S RNA synthesis but did not inhibit either GA-RNA or amylase synthesis. ABA completely inhibited both enzyme synthesis and GA-RNA synthesis. The selectivity of these effects makes a very strong case for the production of GA-RNA being closely correlated with amylase synthesis.

A number of studies have shown that GA treatment causes the production, probably by *de novo* synthesis, of several hydrolases in aleurone tissue and therefore we should be equating GA-RNA and production of all of the enzymes. Our limited evidence (9, 10) indicates that at least most of GA-induced enzymes are controlled as a unit and that assay of one enzyme is probably a good indication of the *titer* of all of them. It seems legitimate therefore to compare a percentage reduction in GA-RNA with a percentage reduction in α-amylase taken as an approximate measure of the reduction in all the GA-induced enzymes.

The synthesis of all of the GA-induced enzymes must require messengers of different molecular weights and taken together these would yield polydisperse RNA on acrylamide gels. Therefore the GA-RNA could be messenger RNA. We have determined the monomeric molecular weights of GA-induced enzymes released from the aleurone tissue and found them to range from 10,000 to 50,000. Messengers for these proteins would range from about 7S to 16S which does not cover the whole range of GA-RNA, but whether the enzymes released from the tissue represent the only GA-induced proteins in aleurone is not known. While we recognize that incorporation of label into GA-RNA could represent (*a*) a promotion of the rates of syntheses of existing RNA species, (*b*) an initiation of syntheses of new species, or (*c*) stabilization of RNA species which are already being synthesized, we are unable to distinguish between these possibilities at this stage.

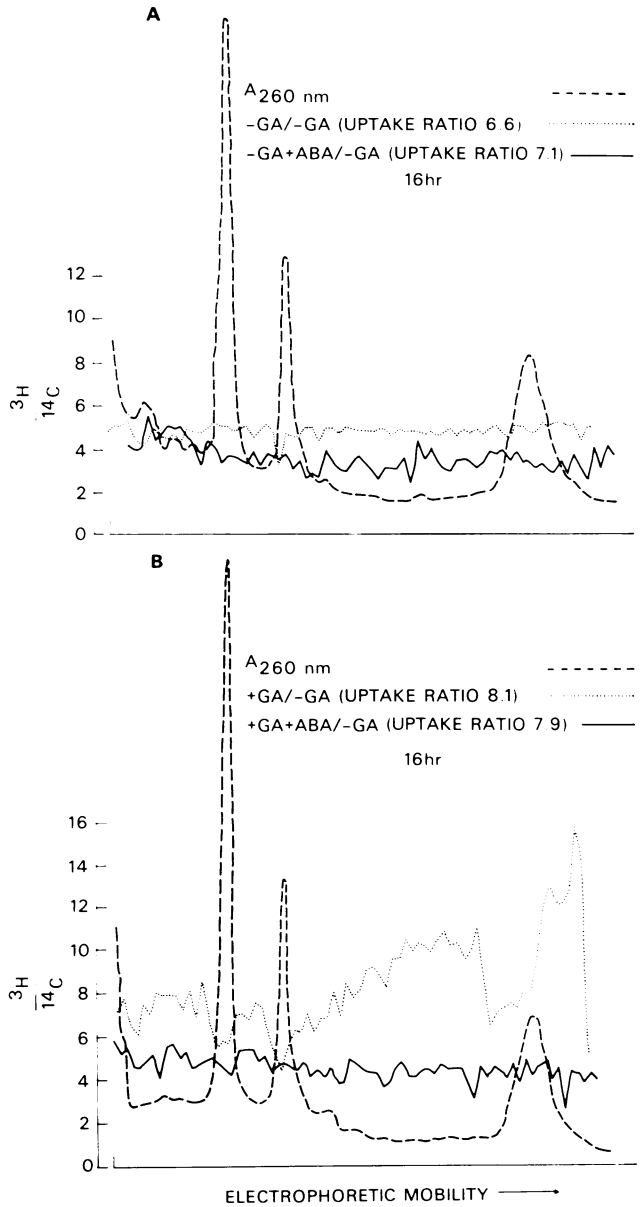

FIG. 7. As in Figure 5 with ABA replacing Act D.

The technique of double labeling of RNA has been applied to the GA effect on barley aleurone before by Chandra and Duynstee (2), but because these workers fractionated their RNA on MAK columns, we are unable to compare the two studies. Nevertheless, they did find increased ratios in their 28S II fraction, a shoulder on the heavy ribosomal RNA peak, and in the DNA-RNA region. We are endeavoring to establish correspondence between the two fractionation systems.

We have found that GA stimulates the production of polydisperse RNA and have established correlations between the production of this GA-RNA and appearance of α-amylase. Our results are consistent with the hypothesis that GA-RNA is at least in part the messenger for α-amylase and other GA-induced enzymes.

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