Plant Physiol. (1973) 52, 208–214

Hormonal Control of Orthophosphate Incorporation into Phospholipids of Barley Aleurone Layers[1]

Received for publication August 11, 1972

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ABSTRACT

Gibberellic acid added to isolated barley aleurone layers enhances orthophosphate incorporation into chloroform-methanol-soluble compounds. The effect is measurable at 4 to 6 hours after the addition of gibberellic acid and reaches a maximum after 8 to 12 hours. The increase in the rate of orthophosphate incorporation is 3- to 5-fold over the rate in control layers incubated without gibberellic acid.

The gibberellic acid enhancement of the rate of phospholipid labeling is inhibited within 1 to 2 hours by cycloheximide, 6-methylpurine, and abscisic acid.

The increase in labeling of phospholipids occurs throughout the subcellular fractions rather than being restricted to a specific fraction or organelle. The increase in radioactivity in phospholipids as shown by thin layer chromatography is due to a proportional increase in all phospholipids.

The enhancement by gibberellic acid of the rate of phospholipid labeling may be required for the subsequent production of gibberellic acid-induced hydrolases.

The seeds of the Gramineae consist of an embryo and an endosperm; the endosperm is composed of an inner starchy material surrounded by a layer of aleurone cells one to five cells thick. In barley, gibberellin-like substances secreted by the embryo during germination cause liquification of the endosperm (22, 30). Aleurone layers, whether as part of intact, embryoless half-seeds or isolated from the endosperm, will respond to added gibberellic acid by synthesizing and secreting several hydrolytic enzymes (3, 5, 22, 29, 30). Thus, the mobilization of reserves in the endosperm is under control of the germinating embryo via gibberellins (21, 24, 31).

There is an 8- to 10-hr lag period (5) between the addition of GA to aleurone layers and the initiation of *de novo* synthesis of α-amylase (11) and protease (13). Examination of events occurring during this lag period will aid in the understanding of the effects of GA on the aleurone layer and the processes required for the ultimate synthesis of GA-induced hydrolases.

During the lag period preceeding α-amylase synthesis there are several events controlled by GA which are directly related to protein synthesis. Jones (16) observed an increase in the amount of rough ER[3] in electron micrographs of aleurone layers treated with GA for 10 hr. Evins and Varner (9) showed an increased incorporation of choline *in vivo* into a semipurified microsomal pellet starting after 4 hr of GA treatment. Two enzymes, phosphorylcholine-cytidyl transferase and phosphorylcholine-glyceride transferase, of the CDP-choline pathway for lecithin synthesis show increased activity within 2 hr of the addition of GA, reaching a maximum after 12 hr of GA treatment (14). Finally, enhanced polyribosome formation and an increase in the total number of ribosomes were observed after 4 to 5 hr and reached a maximum within 10 to 15 hr of GA treatment (8).

Preliminary experiments showed that GA also enhanced the incorporation of ^{32}Pi into chloroform-methanol-soluble components of aleurone layers. Phosphate is a general label for all phospholipids and proved to give consistent results. Thus a detailed study of the control of phospholipid labeling by ^{32}Pi was undertaken to further define processes required for GA-dependent hydrolase production.

MATERIALS AND METHODS

Preparation of Aleurone Layers. Aleurone layers were prepared from barley half-seeds (*Hordeum vulgare* L. cv. Himalaya; seeds from the 1969 harvest supplied by the Agronomy Club, Washington State University, Pullman, Wash.) essentially following the methods of Chrispeels and Varner (5). In most experiments duplicate samples of 10 layers were shaken in a 25-ml Erlenmeyer flask with 2 ml of incubation medium. In some cases 20 to 30 layers were incubated in a 50-ml flask with 5 to 6 ml of incubation medium. The incubation medium contained 1 mM Na acetate buffer, pH 5.0; 20 mM $CaCl_2$; 20 μg/ml chloramphenicol; and 1 μM GA_3 where appropriate. Incubations were carried out at 25 C on a reciprocal shaker. Sterile conditions were maintained during all manipulations.

Incorporation of ^{32}Pi. Carrier-free [^{32}P]H_3PO_4 in 0.1 N HCl purchased from International Chemical and Nuclear Corporation was used in all experiments. During incubation, aleurone layers were labeled by adding 100 to 150 μc of ^{32}Pi directly

[1] This research was supported by the United States Atomic Energy Commission under Contract AT(11-1)-1338 and by Grant GB-8774 from the National Science Foundation to J. E. V. This manuscript is based on a dissertation submitted to Michigan State University by D. E. K. in partial fulfillment of the requirements for the Ph.D. degree. This is Journal Article 6022 from the Michigan Agricultural Experiment Station.

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[3] Abbreviations: ER: endoplasmic reticulum; ^{32}Pi: (^{32}P)-orthophosphate.

to incubation flasks. A labeling period in the range of 30 to 90 min gave a linear accumulation of ^{32}Pi in phospholipids. Early experiments showed that values for uptake of ^{32}Pi were almost independent of time of labeling or phosphate concentration over a wide range of values. That is, there seemed to be an immediate adsorption of large amounts of radioactivity to the layers, which could mask significant differences in the amounts of metabolically available phosphate actually inside the cells. It was found that a 30-min incubation in nonlabeled phosphate following a ^{32}Pi pulse allowed the exchange of adsorbed radioactivity with the medium, thereby reducing measured uptake values to meaningful levels. Thus, the rate of incorporation of ^{32}Pi into phospholipids was routinely measured with a 45-min pulse. Then layers were rinsed in sterile 50 mM KH_2PO_4 and incubated an additional 30 min in new 50 mM KH_2PO_4. At this point, the layers were rinsed again in 50 mM KH_2PO_4 and immediately ground or quick-frozen for homogenization at a later time. Freezing the layers did not affect extraction of labeled compounds. A few experiments used 30 min for labeling and 15 min for the chase. There are noted as they occur.

Determination of Incorporated Radioactivity. After the labeling and washing procedure, aleurone layers were homogenized in a mortar with sand and grinding buffer (0.1 M HEPES, pH 7.55, and 0.45 M sucrose). The homogenate was centrifuged at 4,000*g* for 10 min followed by a centrifugation at 10,000*g* for 15 min. This procedure gave a cleaner final supernatant than could be obtained from just one centrifugation as well as providing a crude fractionation of the homogenate.

Four determinations were made on the final supernatant: (*a*) aliquots were counted directly for total uptake; partitioned to separate (*b*) ^{32}Pi from (*c*) phosphate-containing organic compounds; and extracted to yield (*d*) the phospholipid fraction.

Radioactivity in organic phosphates was assayed by the method of Saha and Good (27). This procedure results in the partitioning of all inorganic phosphate into a butanol-benzene phase as a phosphomolybdate complex. Therefore, all radioactivity in the aqueous phase can be attributed to ^{32}Pi incorporated into organic compounds (including pyro- or polyphosphates). The exact nature of these organic phosphates was not determined, but it is assumed that they include nucleotides, sugar phosphates, etc. Not included are the phospholipids which partition into the butanol-benzene phase, or RNA and phosphoproteins which are precipitated and removed by a filtration step. In this system, however, these compounds represent only a small percentage of the total radioactivity measured in either the upper or lower phase. Counting the butanol-benzene phase gives an estimate of ^{32}Pi in the aleurone layers. It was found that the ratio, ^{32}Pi uptake, was fairly constant throughout any given experiment. Therefore, results are expressed in terms of uptake rather than ^{32}Pi even though both measurements were made. Uptake includes incorporated ^{32}P as well as ^{32}Pi and gives a better estimate of the total amount of ^{32}Pi entering the cells.

Phospholipids were isolated following the procedures of Folch *et al.* (12). Usually 1 ml of supernatant was vigorously extracted with 4 to 5 ml of chloroform-methanol (2:1, v/v). The upper phase and protein at the interface were removed by suction. The lower (chloroform) phase was washed three times with upper phase solvents (chloroform-methanol-water, 3:48:47, v/v) which also contained 0.8% NaCl and 0.2% $MgCl_2$ to prevent any further partitioning of phospholipids into the upper phase wash. It was determined that three washes were sufficient to break emulsions in the chloroform phase and to remove all nonphospholipid ^{32}P trapped in that manner. The chloroform phase was either concentrated for TLC or transferred to a scintillation vial and dried for counting.

Thin Layer Chromatography. Phospholipids were separated by TLC for estimation of radioactivity in individual compounds. Precoated 20- × 20-cm TLC glass plates with a 0.25-mm silica gel layer (E. Merck, Darmstadt, Germany) were purchased from Brinkmann Instruments. The plates were developed in a two-dimensional system of Rouser *et al.* (26). In the first direction chloroform-methanol-14 N NH_4OH (65:35:5, v/v) was used. After drying, the second dimension was developed in chloroform-acetone-methanol-acetic acid-water (100:40:20:20:10, v/v).

Visualization was accomplished first with iodine vapor and then by several specific sprays: the Dragendorf reagent for choline-containing phospholipids (2); the ninhydrin spray for phosphatidyl ethanolamine and phosphatidyl serine; and the molybdenum blue reagent for all phospholipids (28). Identifications were confirmed by comparing the migration of sample compounds to that of authentic standards (Supelco, Inc.). Individual spots were scraped into scintillation vials for measurement of ^{32}Pi incorporation.

Phosphorus Analysis. The chemical analysis of the phosphorus content of phospholipid and organic phosphate fractions were carried out using the method of Bartlett (1). Appropriate amounts of an orthophosphate standard were carried through the procedure for conversion of absorbance to micromoles of Pi.

Measurement of Radioactivity. The scintillation fluid used for all measurements was a mixture of toluene-Triton X-100 (2:1, v/v) which contained 4 g of PPO and 0.1 g of dimethyl POPOP per liter of toluene (23). This mixture has the advantage of being able to solubilize phospholipids as well as adequately emulsify aqueous samples.

RESULTS

Time Course of Phospholipid Labeling. Figure 1 shows a typical time course of the rate of ^{32}Pi incorporation into phospholipids in the presence and absence of GA. After 4 hr the rate of incorporation of ^{32}Pi into phospholipids increases rapidly, reaching a maximum 8 to 12 hr after addition of GA.

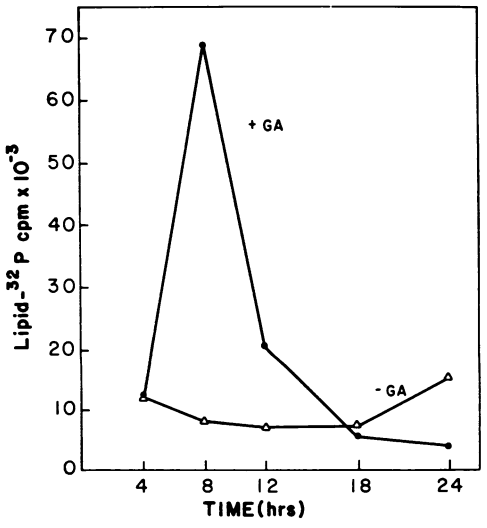

FIG. 1. Time course of the rate of ^{32}Pi incorporation into phospholipids. Ten aleurone layers were pulse-labeled at the indicated times, and ^{32}Pi incorporation into the phospholipids of the 10,000*g* supernatant was measured.

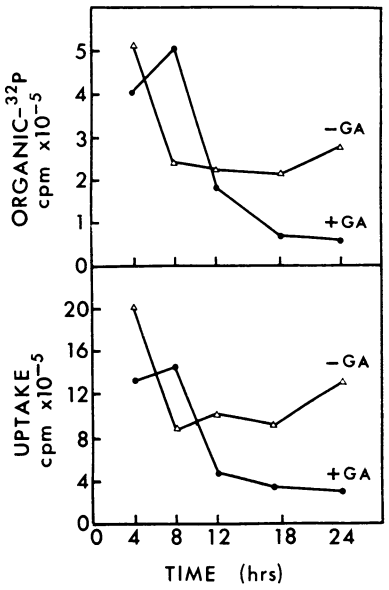

FIG. 2. Time course of ^{32}Pi uptake into aleurone layers (lower) and incorporation of ^{32}Pi into phosphate-containing organic compounds (upper). The data are from the same experiment as Figure 1.

The rate of incorporation then decreases and approximates the level of the −GA control by 18 to 24 hr.

Some phospholipid labeling also occurs in −GA tissue. The rate of labeling is relatively constant for 18 hr and increases somewhat by 24 hr.

The uptake of ^{32}Pi varies during the 24-hr time course in both control and GA-treated aleurone layers (Fig. 2, lower). The usual pattern is that uptake in hormone-treated layers is equal to or greater than uptake in control layers at early times but decreases to much lower levels with longer incubation periods.

These changes in uptake are directly reflected in the incorporation of ^{32}Pi into phosphate-containing organic compounds (Fig. 2, upper). In addition, the relative rates of ^{32}Pi incorporation into phospholipids are probably affected in a similar manner. Therefore, we decided that a more accurate measurement of phospholipid labeling could be obtained by expressing. ^{32}Pi incorporation into phospholipids as a percentage of ^{32}Pi incorporation into the total organic phosphate fraction. In this way, ^{32}Pi incorporation into phospholipids is adjusted for differential rates of labeling of the organic phosphate pools, whether such differences are due to uptake or various metabolic effects. Assuming a single ^{32}Pi pool and a single organic phosphate pool, an increase in the ratio of phospholipid radioactivity to organic phosphate radioactivity indicates a true increase in the rate of phospholipid labeling since all immediate phospholipid precursors are a part of or are derived from what is measured as organic phosphate.

When the data are expressed in this manner (Fig. 3), the time course is only slightly different from that of Figure 1. The rate of phospholipid labeling in GA-treated layers still has a maximum at 8 hr, but the decrease is more gradual and approaches the level of the control layers only after 24 hr.

The maximum effect of GA is usually 3- to 5-fold when data are expressed as a percentage whereas the absolute incorporation of ^{32}Pi into phospholipids may increase 7- to 10-fold or more due to increased ^{32}Pi uptake. Thus, the phospholipid to organic phosphate ratio actually gives a more conservative estimate of the enhancement by GA of phospholipid labeling.

There is variability in the phospholipid to organic phosphate ratio obtained from different experiments. Incubation of half-seeds instead of isolated aleurone layers and the lengths of the pulse and chase periods also affect this ratio. Regardless of the numerical value obtained, however, the enhancement by GA of phospholipid labeling falls in the range of 3- to 5-fold within any one experiment.

Characterization of ^{32}Pi Incorporation. To be sure that the GA enhancement of phospholipid labeling was not due to an artifact of grinding or fractionation, all fractions obtained by differential centrifugation of an aleurone layer homogenate were examined. The 4,000*g* and 10,000*g* pellets as well as the supernatant from plus and minus GA treatments were extracted with chloroform-methanol, and the phospholipids were counted. Table I shows that the GA enhancement of ^{32}Pi incorporation into phospholipids occurs in all three cell fractions. The 4,000*g* pellet which contains unbroken cells, cell debris, and nuclei; the 10,000*g* pellet which is enriched for mitochondria; and the supernatant which contains the microsomal fractions all show an 11- to 12-fold stimulation of incorporation by GA. Within the −GA or +GA treatments, the relative distribution of radioactivity among the fractions is constant.

Results show that the effect of GA on the incorporation of ^{32}Pi into phospholipids in the supernatant fraction is not an artifact due to better homogenization of +GA layers or a differential distribution of radioactivity among fractions of different treatments.

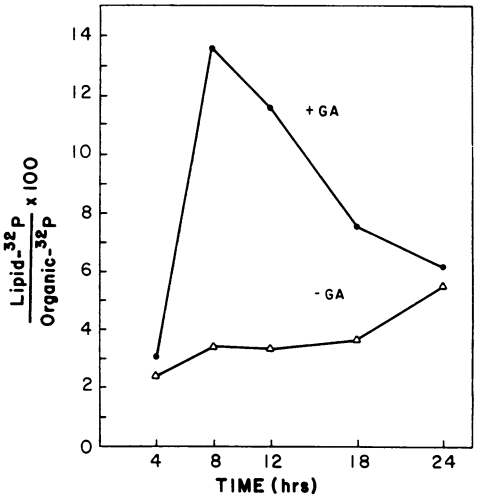

FIG. 3. Time course of phospholipid labeling. Incorporation of ^{32}Pi into phospholipids is expressed as a percentage of the ^{32}Pi incorporation into organic phosphates. The data are taken from Figures 1 and 2.

Table I. *Distribution of Labeled Phospholipids in Different Cell Fractions*

Twenty aleurone layers per flask were incubated for 8 hr with or without 1 μM GA_3. At that time they were labeled with 225 μc of ^{32}Pi per flask for 30 min and chased with 50 mM KH_2PO_4 an additional 15 min.

Cell Fraction	−GA		+GA		Enhancement by GA
	cpm	*% of total*	*cpm*	*% of total*	
4,000*g* pellet	26,000	68	310,000	69	11.9×
10,000*g* pellet	4,800	13	55,000	12	11.5×
Supernatant	7,400	19	86,000	19	11.6×

Other experiments demonstrated the particulate nature of the phospholipids from the 10,000*g* supernatant. It was found that 80 to 90% of the chloroform-methanol-soluble ^{32}P in the 10,000*g* supernatant was pelleted by centrifugation at 105,000*g* for 1 hr. Furthermore, 95% of the pelleted radioactivity was solubilized by treatment with 0.1% Triton X-100 and was not repelleted by a second centrifugation. This indicates that the phospholipid radioactivity examined in the 10,000*g* supernatant is membrane-localized.

Final confirmation of the assumption that chloroform-methanol-soluble ^{32}P-radioactivity from various cell fractions actually represents phospholipids comes from two-dimensional TLC of the chloroform-methanol extracts. Sprays specific for phosphate, amino groups, and choline as well as chromatography of authentic standards gave a positive identification of the phospholipids. Individual phospholipids were located both chemically and by the presence of radioactivity. Over 90% of the radioactivity on each TLC plate could be attributed to known phospholipids.

The GA-enhanced increase in radioactivity seemed to be distributed among most of the labeled phospholipids (Table II). Small increases in the relative amounts of radioactivity in phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl inositol at the expense of phosphatidyl glycerol have occurred after 10 hr of GA treatment. The amount of radioactivity in phosphatidic acid is not consistent and is probably due to degradation of other phospholipids. On the whole, hormone treatment did not drastically affect the percentage of the total radioactivity for any one phospholipid but rather resulted in a proportionate increase in labeling of all phospholipids (except phosphatidyl glycerol).

Effects of Metabolic Inhibitors. The GA-induced enhancement of α-amylase synthesis is inhibited to varying degrees by several metabolic inhibitors (5, 6). Cycloheximide completely inhibits α-amylase synthesis almost immediately, while the inhibition by actinomycin D is less severe.

Figure 4 shows that phospholipid labeling is also rapidly inhibited by cycloheximide. One hour after the addition of cycloheximide, the rate of phospholipid labeling in +GA control layers has increased, but this increase is prevented in layers treated with cycloheximide.

Another type of experiment shows that for short treatment periods (up to 2 hr), cycloheximide inhibits GA-enhanced capacity for phospholipid labeling but does not affect phospholipid labeling *per se* in control layers (Table III).

Table II. *Relative Distribution of Radioactivity in Individual Phospholipids*

Aleurone layers were incubated with or without GA for 10 hr and pulse-labeled with ^{32}Pi. Total phospholipids were analyzed by grinding the layers directly in chloroform-methanol followed by TLC of the washed extracts. Approximately 3,000 cpm and 25,000 cpm were analyzed for −GA and +GA samples, respectively. The compounds listed below account for 97 and 92%, respectively, of the radioactivity which was analyzed.

	Radioactivity Incorporated	
	−GA	+GA
	%	%
Phosphatidyl choline	37	43
Phosphatidyl ethanolamine	13	18
Phosphatidyl inositol	14	20
Phosphatidyl glycerol	17	6
Cardiolipin (tentative)	14	13
Phosphatidic acid	5	0

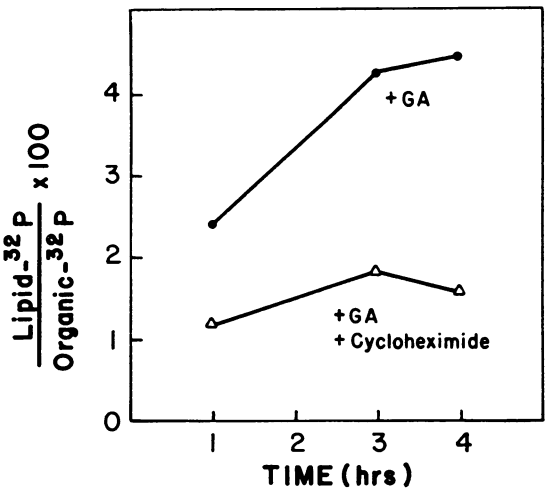

FIG. 4. Time course of inhibition of phospholipid labeling by cycloheximide. Half-seeds were preincubated in GA for 10 hr. At 0 time on the graph aleurone layers were removed from the half-seeds and cycloheximide (10 μg/ml final concentration) was added to one set of flasks. At subsequent intervals layers were labeled with ^{32}Pi for 30 min followed by a 15-min chase and phospholipid extraction.

Table III. *Cycloheximide Inhibition of Phospholipid Labeling in Control and GA-treated Aleurone Layers*

Aleurone layers were incubated as described under "Treatment." All treatments were labeled with ^{32}Pi after 7 hr of incubation. Phospholipids were extracted and counted.

Treatment	Radioactivity in Phospholipids	
	−GA	+GA
	cpm	
Control (7-hr incubation)	850	5100
Cycloheximide added at hr 5	1250	2500
Cycloheximide added at 0 time	220	150

These experiments indicate that there is a requirement for continued protein synthesis, in order for a GA enhancement of phospholipid labeling to take place. Cycloheximide specifically inhibits hormone-induced increases in the rate of phospholipid labeling within 1 hr. On the other hand, the possibility does exist that the inhibition of ^{32}Pi incorporation into phospholipids by cycloheximide is due to indirect effects of the drug, rather than a direct inhibition of protein synthesis.

In a similar experiment, 6-methylpurine was also shown to inhibit GA-enhanced phospholipid labeling (Fig. 5). While the rate of phospholipid labeling continues to increase in layers treated with only GA, 6-methylpurine has inhibited most of this increase after 2 hr, and after 4 hr the inhibition is even greater.

Another inhibitor of RNA synthesis, actinomycin D, also inhibits GA-enhanced phospholipid labeling (Table IV). When actinomycin D is added at the same time as GA, concentrations of 100 and 50 μg/ml inhibit phospholipid labeling, but there may be a slight stimulation at 25 μg/ml. The extent of inhibition is approximately the same as was found for α-amylase production when measured after 24 hr (5).

The effects of RNA synthesis inhibitors on phospholipid

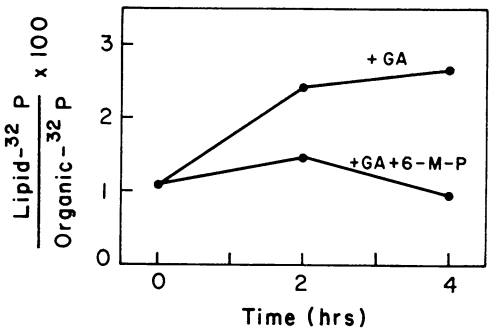

FIG. 5. Inhibition of phospholipid labeling by 6-methylpurine. Aleurone layers were removed from half-seeds after a 7-hr incubation in GA. Incubations were continued in GA with or without 1 mM 6-methylpurine. At 0, 2, and 4 hr layers were pulsed 30 min with ^{32}Pi followed by a 15-min chase and phospholipid extraction.

Table IV. *Effect of Actinomycin D on Phospholipid Labeling*

Isolated aleurone layers were incubated 9 hr in the presence o GA and actinomycin D at the indicated concentrations. At that time the layers were pulse-labeled and phospholipids were extracted.

Treatment	Lipid-^{32}P / Organic-^{32}P
	%
−GA	1.7
+GA	6.4
+GA	
+ actinomycin D (25 μg/ml)	8.4
+ actinomycin D (50 μg/ml)	4.4
+ actinomycin D (100 μg/ml)	3.3

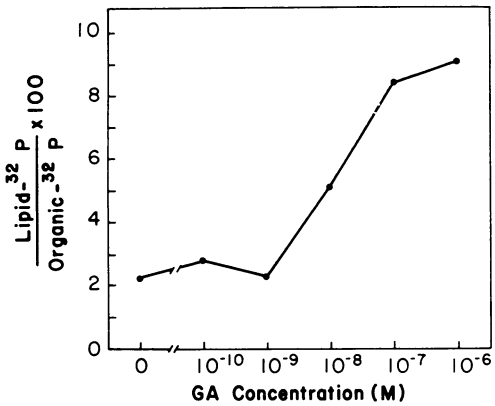

FIG. 6. The response of phospholipid labeling to increasing concentrations of GA. Aleurone layers were incubated in solutions of the indicated GA concentration for 10 hr. They were then pulse-labeled with ^{32}Pi, and phospholipids were extracted.

labeling correspond to the effects on α-amylase synthesis; *i.e.*, 6-methylpurine is a more effective inhibitor than actinomycin D and is effective when added during the mid-course of GA action. These results suggest a requirement for RNA synthesis in order to obtain GA-enhanced phospholipid labeling. However, the possibility of interference with other metabolic processes resulting in a decreased rate of phospholipid labeling cannot be ruled out.

Hormonal Control. The response of phospholipid labeling to increasing concentrations of GA is shown in Figure 6. Above 10^{-9} M GA phospholipid labeling increases with an increase in the GA concentration. A maximum is reached at 10^{-6} M. Although the response is not proportional over as wide a range of GA concentrations as is α-amylase release (17), there is a clear dependence of the rate of phospholipid labeling on GA concentration.

Abscisic acid inhibits the GA-induced production of α-amylase in barley aleurone layers (6). It also inhibits the GA-enhanced incorporation of ^{32}Pi into phospholipids. When ABA is added to GA-treated layers, any further enhancement of the rate of phospholipid labeling is prevented (Fig. 7). Other experiments showed no effect of ABA on the basal rate of phospholipid labeling in −GA control layers, whether ABA was added at 0 time or several hours after the addition of GA.

The effect of increasing concentrations of ABA on the rate of phospholipid labeling is shown in Figure 8. There is no inhibition of the GA response until the ABA concentration reaches 0.1 μM. At 1.0 μM, phospholipid labeling has been reduced to the level of the −GA layers. The inhibition of phospholipid labeling is sensitive to the concentration of ABA over two orders of magnitude when the ABA is added at 0 time.

Additional Biochemical Data. In an effort to learn more about the nature of the phospholipid labeling enhanced by GA, the incorporation of ^{14}C-acetate by aleurone layers was

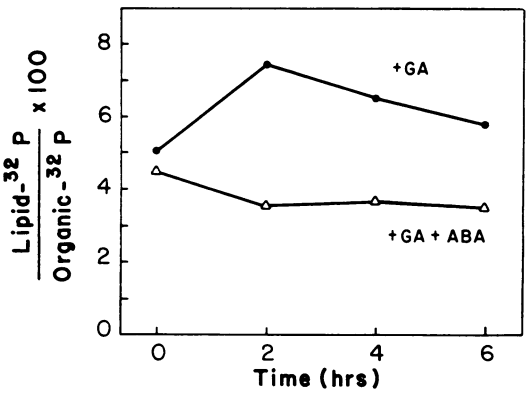

FIG. 7. Inhibition of GA-enhanced phospholipid labeling by abscisic acid. Forty aleurone layers per flask were incubated in 0.1 μM GA. After 8 hr, 2 μM ABA was added to one set of layers (0 time on graph). At the indicated times, 10 layers were removed from duplicate flasks and pulse-labeled with ^{32}Pi, and phospholipids were extracted.

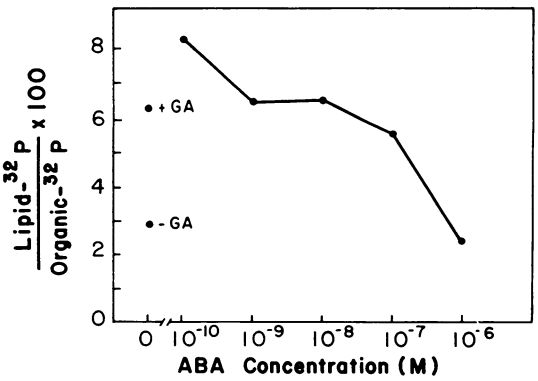

FIG. 8. Progressive inhibition of phospholipid labeling by increasing concentrations of ABA. Aleurone layers were incubated for 8 hr with both 1.0 μM GA and ABA at the indicated concentrations present from the start. The layers were then pulse-labeled with ^{32}Pi, and phospholipids were extracted.

Table V. *Incorporation of ^{14}C-Acetate by Aleurone Layers*

After incubation for 9 hr in medium minus acetate buffer, aleurone layers were labeled with 2 μc of Na acetate-2-^{14}C (85 mc/mmole) per flask for 1 hr. After the regular homogenization procedure, aliquots of the supernatant were counted for uptake, extracted with chloroform-methanol, or precipitated with 10% trichloroacetic acid and filtered through a nitrocellulose filter.

	−GA	+GA
	cpm × 10^{-4}/10 layers	
Chloroform-methanol-soluble	7.1	7.5
Trichloroacetic acid-insoluble	14.9	14.3
Uptake of ^{14}C-acetate	60.3	73.5

Table VI. *Phospholipid Content of Aleurone Layers*

Phospholipids were extracted from the 4,000*g* and 10,000*g* pellets and supernatant of aleurone layer homogenates. The values below are sums of these three determinations.

Time	Phospholipid-P	
	−GA	+GA
hr	*μmoles/ 10 layers*	
6	0.67	0.72
12	0.73	0.66
18	0.67	0.67
24	0.70	0.51

examined. Table V shows that there was no effect of GA on the incorporation of acetate into compounds soluble in chloroform-methanol or insoluble in trichloroacetic acid. These categories represent lipids, and proteins and membranes, respectively. Thus, the stimulation of incorporation of ^{32}Pi into phospholipids is not accompanied by a similar increase in fatty acid synthesis from an acetate precursor.

Chemical determinations of phospholipid levels in aleurone layers were also carried out (Table VI). When the separate cell fractions were analyzed, the relative distribution of phospholipids in the pellets and the supernatant resembled the distribution of radioactivity in these fractions (Table I). That is, the 4,000*g* pellet, the 10,000*g* pellet, and the 10,000*g* supernatant contained 65, 14 and 21%, respectively, of the total phospholipid phosphorus in the cell. There were no effects of incubation time or GA on this distribution.

Data presented in this section suggest that the increase in ^{32}Pi incorporation into phospholipids does not involve net synthesis of phospholipids but occurs during turnover of existing lipid or phospholipid components.

DISCUSSION

To study the control of phospholipid synthesis it must first be established that: (*a*) the radioactivity measured is actually incorporated into phospholipids; and (*b*) a change in the rate of incorporation of ^{32}Pi into phospholipids is an accurate measurement of phospholipid labeling and is not merely a reflection of variations in the uptake or metabolism of ^{32}Pi.

The first point is adequately covered by the extraction and characterization procedures. Chloroform-methanol extraction followed by a wash of the chloroform phase to break emulsions insures that only ^{32}P in phospholipids is counted. Thin layer chromatography of extracts verified that all ^{32}P present was incorporated into phospholipids.

To insure that the measured rates of ^{32}Pi incorporation into phospholipids were representative of *in vivo* metabolism, ^{32}Pi uptake and incorporation into organic phosphates were also monitored. Since all ^{32}P incorporated into phospholipids will have passed through the organic phosphate pool, this pool represents the precursor pool of ^{32}P for phospholipid synthesis. Therefore, the incorporation of ^{32}Pi into organic phosphates can be used as an internal standard to estimate the level of ^{32}P which is available for phospholipid labeling. Use of the ratio, phospholipid ^{32}P to organic ^{32}P, corrects the level of radioactivity in phospholipids for any differences in labeling of the organic phosphate pools. The result is an accurate measurement of the relative rate of phospholipid labeling. All variations in ^{32}Pi uptake, internal pools of ^{32}Pi, and the rate of incorporation of ^{32}Pi into organic compounds are taken into account by such a calculation.

The enhancement by GA of the rate of incorporation of ^{32}Pi into phospholipids can now be added to the series of events which precede α-amylase production in barley aleurone layers. The response is initiated 4 to 6 hr after the addition of GA and increases to a maximum after 8 to 12 hr. This time course is in good agreement with the work of Evins (8) which showed GA-enhanced polysome formation and an increase in the number of ribosomes over the same time period. Thus there is a concomitant increase in two cellular components having a major role in protein synthesis. The decline in the rate of phospholipid labeling occurs during the time when the amount of polysomes has also reached a plateau and a linear rate of α-amylase production has been established.

The work of Collins *et al.* (7) showed an increase in the specific activity of ^{32}Pi-labeled CTP 30 and 90 min after the addition of GA to wheat aleurone layers. This implies a more rapid turnover of CTP at early incubation times with GA. Since CTP has a fundamental role in phospholipid synthesis, this effect could be related to the enhancement of phospholipid labeling observed in barley. However, there is a discrepancy in the timing of the events, since the GA effect on the specific activity of CTP has disappeared by 2 hr while increases in phospholipid labeling do not begin until after 4 hr of GA treatment.

A possible role for the increased ^{32}Pi incorporation into phospholipids during the subsequent production of α-amylase and other hydrolases is suggested by the metabolic and hormonal controls. Cycloheximide rapidly inhibits any GA-enhanced increase in the rate of phospholipid labeling without affecting the basal rate in control tissue. The same is true of 6-methylpurine.

The function of ABA as an antagonist of GA action has been documented for lettuce seed germination (18), germination of hazel seeds (25), and in the aleurone layer system for inhibition of α-amylase production (4), polysome formation (10), and production of lecithin-synthesizing enzymes (14). Abscisic acid also causes a rapid and specific inhibition of the GA enhancement of the rate of phospholipid labeling. This inhibition is dependent on ABA concentration. Complete inhibition is obtained at 1 μM ABA, which means that phospholipid labeling is more sensitive to ABA than seed germination and is as sensitive as other processes in aleurone layers cited above.

It has also been shown (20) that the rate of ^{32}Pi incorporation into phospholipids is inhibited by osmotic stress. The inhibition closely resembles the degree of inhibiton of α-amylase production by mannitol (15) and again is specific for the GA-induced increase in the rate of phospholipid labeling.

Attempts at mimicking the stimulation by GA of phospholipid labeling have failed. Cyclic AMP did not enhance the

rate of phospholipid labeling in the presence or absence of GA (19). The addition of amino acids to the incubation medium likewise will not substitute for GA with respect to either α-amylase production or phospholipid labeling (Varner and Koehler, unpublished results).

There was no indication of a specific stimulation of phospholipid labeling associated with the microsomal fraction of the aleurone cells. Phospholipid radioactivity had the same enhancement by GA in the 4,000*g* pellet, the 10,000*g* pellet, and the 10,000*g* supernatant (Table I). The chemical level of phospholipid phosphorus closely paralleled the content of phospholipid-^{32}P in all three cell fractions. Consequently, the specific activity of the phospholipids remained constant from fraction to fraction. The presumably ER-rich supernatant showed no increase in total phospholipid phosphorus due to GA treatment. There is the possibility of cross contamination of membranes among the cell fractions. Aleurone cells have very thick cell walls, and the severe grinding procedures needed to insure cell breakage could disrupt organelles. Although purified membrane fractions were not examined, these data provide no evidence that synthesis of ER over the time period studied is preferentially enhanced. The GA enhancement of ^{32}Pi incorporation into phospholipids seems to be a general phenomenon throughout the cell. The increased incorporation of radioactivity cannot be attributed to any specific type of membrane or to any individual phospholipid.

The question of whether the enhancement by GA of ^{32}Pi incorporation into phospholipids is due to net synthesis or only increased turnover of phospholipids is still open. The absence of an increase in lipid phosphorus during GA treatment and the lack of a GA stimulation of acetate incorporation into lipids suggest that there is a GA-enhanced turnover rather than a net synthesis of phospholipids. On the other hand, the increased labeling of phospholipids could represent a small increase in the chemical level of phospholipids which is not readily detectable.

GA may cause the mobilization of phospholipids from storage to support membrane synthesis. If the mechanism of mobilization results in a partial degradation of the phospholipid molecule, ^{32}P could be incorporated into phospholipids during resynthesis. This would account for the lack of an increase in total lipid phoshporus or in acetate incorporation.

Acknowledgments—The authors thank R. Mense for technical assistance and Dr. H. Kende for helpful discussions.

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