HORMONAL CONTROL OF PHOSPHOLIPID SYNTHESIS IN BARLEY ALEURONE LAYERS

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IN BARLEY ALEURONE LAYERS

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ABSTRACT

HORMONAL CONTROL OF PHOSPHOLIPID SYNTHESIS
IN BARLEY ALEURONE LAYERS

By

Don Edward Koehler

Gibberellic acid (GA_3) enhances the rate of phospholipid synthesis in barley aleurone layers. Using ^{32}Pi incorporation into chloroform-methanol soluble compounds as an assay for the response, enhancement was shown to start at 4-6 hr after the addition of GA and reached a maximum after 8-12 hr. The increase in the rate of ^{32}Pi incorporation was 3-5 fold over the rate in control layers incubated without GA.

The GA enhancement of the rate of phospholipid synthesis could be inhibited within 1-2 hr by cycloheximide, 6-methylpurine, and abscisic acid.

The ratio, organic-^{32}P : uptake of ^{32}Pi, was enhanced 50% by treatment with GA. An osmotic stress imposed on the layers by incubating them in mannitol inhibited the rate of phospholipid synthesis but not the increase in the organic-^{32}P : uptake ratio.

Removal of GA from the layers resulted in a decrease in phospholipid synthesis. There was no increase in the rate of phospholipid synthesis when layers were treated with 3', 5'-cyclic AMP.

The increase in labeling of phospholipids occurred throughout the cell rather than being restricted to a specific cell fraction or organelle. The increase in radioactivity in phospholipids is due to a proportional increase in all phospholipids as shown by TLC.

The enhancement of the rate of phospholipid synthesis by GA is thought to be required for the subsequent production of GA-induced hydrolases.

HORMONAL CONTROL OF PHOSPHOLIPID SYNTHESIS IN BARLEY ALEURONE LAYERS

By

Don Edward Koehler

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in partial fulfillment of the requirements

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Department of Biochemistry

1972

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ABBREVIATIONS

ABA	Abscisic Acid
ER	Endoplasmic reticulum
GA	Gibberellic acid (GA_3)
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
^{32}Pi	(^{32}P) orthophosphoric acid
POPOP	1, 4-bis(2-(4-methyl-5-phenyloxazolyl))benzene
PPO	2, 5-diphenyloxazole
TCA	Trichloroacetic acid
TLC	Thin-layer chromatography

INTRODUCTION

The seeds of the Gramineae consist of an embryo, the starchy endosperm, and a layer of aleurone cells enclosing the endosperm. In barley, gibberellin-like substances secreted by the embryo during germination cause liquification of the endosperm (Yomo, 1960; Paleg, 1960). Aleurone layers, whether as part of intact, embryo-less half seeds or isolated from the endosperm, will respond to added gibberellic acid (GA_3) by synthesizing and secreting a series of hydrolytic enzymes (Yomo, 1960; Paleg, 1960; Briggs, 1963; Varner, 1964; Chrispeels and Varner, 1967 a). Thus the mobilization of reserves in the endosperm is under control of the germinating embryo via gibberellins (Yomo and Iinuma, 1966; MacLeod and Palmer, 1966; Radley, 1967).

The barley aleurone layer consists of non-dividing, protein-rich cells three layers thick. Its response to GA when isolated makes this tissue attractive for studying the hormonal induction of enzyme synthesis.

There is an 8-10 hour lag period (Chrispeels and Varner, 1967 a) between the addition of GA to aleurone layers and the initiation of de novo synthesis of α-amylase (Filner and Varner, 1967) and protease (Jacobsen and Varner, 1967).

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.

The results of Siekevitz and Palade (1966) on the synthesis and secretion of pancreatic amylase led to their suggestion that synthesis of proteins destined for export from the cell occurs on the endoplasmic reticulum (ER). Tata (1968) found a correlation in the timing of the hormonal stimulation of RNA, protein, and membrane synthesis in several systems. Thus the study of the aleurone layer in which there is hormonal control of protein synthesis and secretion becomes especially interesting.

During the lag period preceeding α-amylase synthesis there are several events controlled by GA which are directly related to protein synthesis. Jones (1969 b) observed an increase in the amount of rough ER in electron micrographs of aleurone layers treated with GA for 10 hr. Evins and Varner (1971) showed an increased incorporation of choline into a semi-purifed microsomal pellet starting after 4 hours 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 (Johnson and Kende, 1971). Finally, enhanced polyribosome formation and an increase in

the total number of ribosomes was observed after 4-5 hr and reached a maximum within 10-15 hr of GA treatment (Evins, 1971).

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 synthesis was undertaken to further define processes required for GA-effected 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 (1967a). Half seeds were prepared by making two transverse cuts across a barley seed, removing the embryo half and a small portion of the opposite tip of the seed. Half seeds were sterilized in excess 1% NaOCl for 15-20 min, rinsed 5-8 times with sterile distilled water, and incubated for 3 days on sterile moist sand in a foil-wrapped Petri dish. At this time layers were removed from the starchy endosperm with the aid of two spatulas. 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 20-30 layers were incubated in a 50 ml flask with 5-6 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

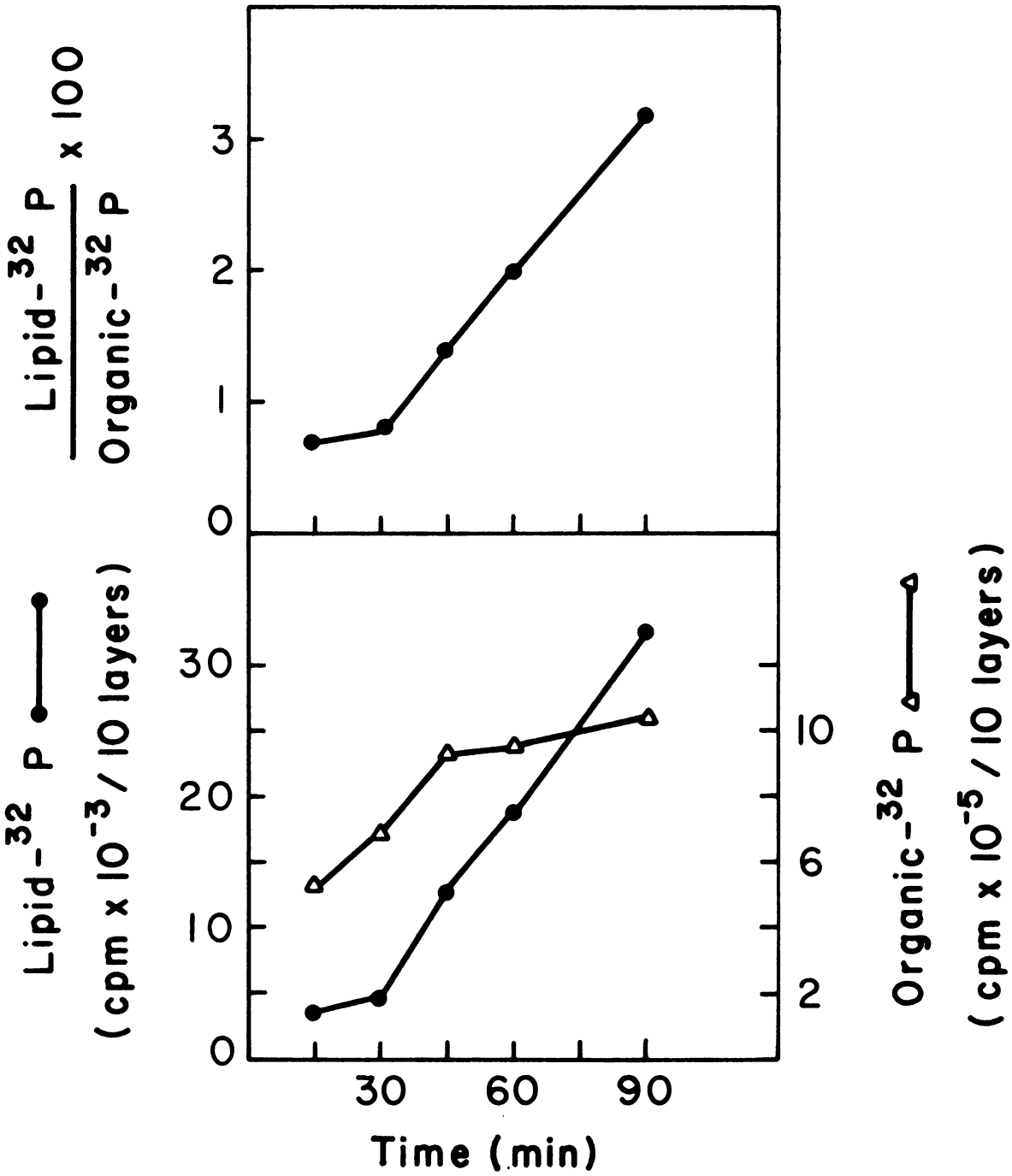
carried out at 25° on a reciprocal shaker. Sterile conditions were maintained during all manipulations.

Incorporation of ^{32}Pi

Carrier free [^{32}P] H_3PO_4 in 0.1 N HCl (hereafter abbreviated ^{32}Pi) purchased from International Chemical and Nuclear Corp. was used in all experiments. During incubations, aleurone layers were labeled by adding 100-150 μc ^{32}Pi directly to incubation flasks. After 45 min, layers were rinsed in sterile 0.05 M KH_2PO_4 and incubated an additional 30 min in new 0.05 M KH_2PO_4. At this point, layers were rinsed again in 0.05 M KH_2PO_4 and immediately ground or quick-frozen for homogenization at a later time. It was experimentally verified that freezing the layers did not affect extraction of labeled compounds.

The choice of times for the labeling period and the chase was made from the experiments shown in Figures 1 and 2. Figure 1 (lower) is the time course of the accumulation of radioactivity in phospholipids and organic phosphates (these fractions are operationally defined in subsequent paragraphs). After 30 min there is a linear accumulation of ^{32}P in phospholipids. Therefore the choice of a time longer than 30 min for pulse labeling should give a good measurement of the rate of phospholipid synthesis. The upper graph in Figure 1 shows that the lipid-^{32}P : organic-^{32}P ratio is also linear after 30 min. This is important because this ratio will also be used to estimate phospholipid synthesis.

Figure 1. Short term accumulation of ^{32}P in lipids and organic phosphates. Duplicate flasks of aleurone layers which had been preincubated in GA for 12 hr were labeled with 140 μc ^{32}Pi. At the indicated times samples of 10 layers were removed from each flask and the radioactivity in the various components was determined.

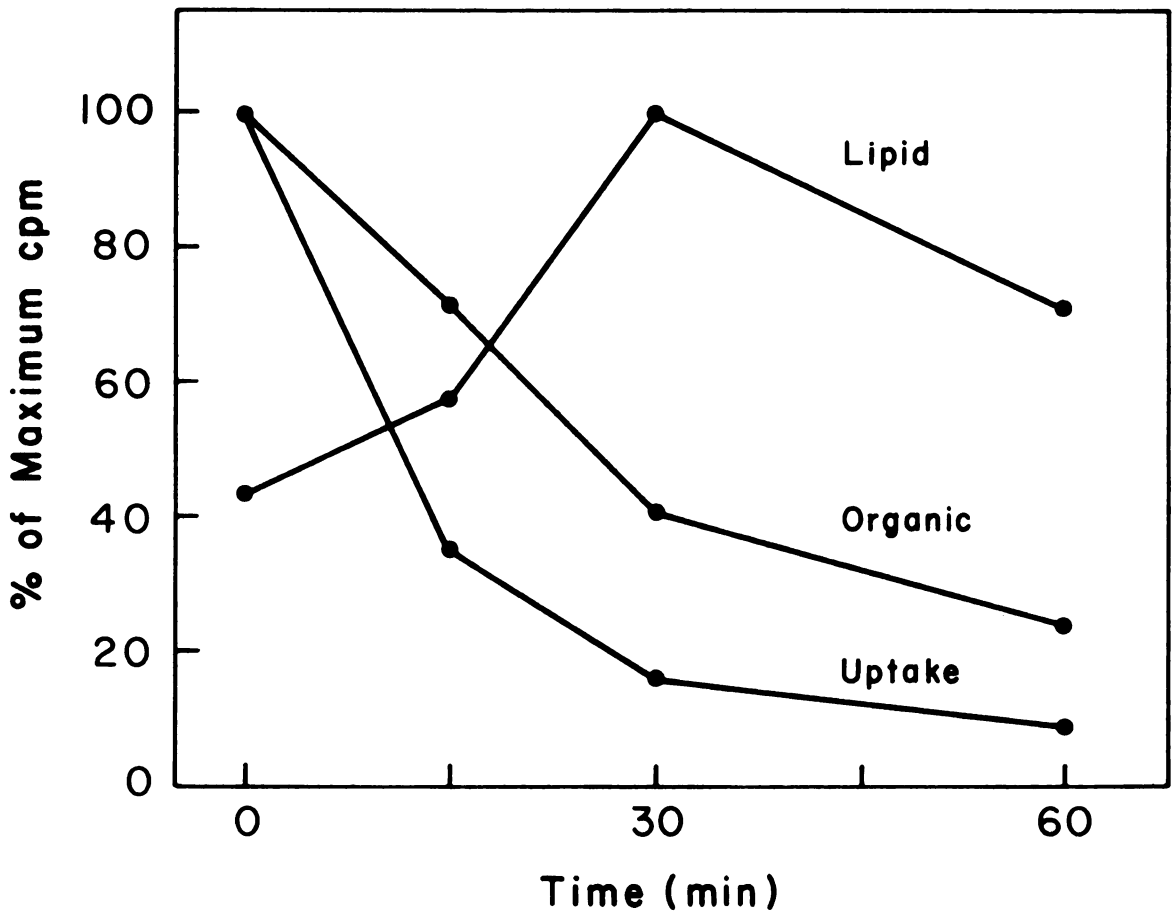

Lipid-32 P / Organic-32 P x 100
0
1
2
3
Lipid-32 P (cpm x 10-3 / 10 layers)
Organic-32 P (cpm x 10-5 / 10 layers)
0
10
20
30
2
6
10
30
60
90
Time (min)

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. Such non-specific binding could be due to calcium phosphate precipitation in the cell walls or the binding of phosphate to other cell wall components. It was found that a short incubation in non-labeled phosphate following a ^{32}Pi pulse seemed to allow the exchange of adsorbed radioactivity with the medium, thereby reducing measured uptake values to meaningful values.

The results of such a chase experiment are shown in Figure 2. Incubation periods up to 30 min result in the most rapid loss of uptake radioactivity but still give accumulation of ^{32}P in lipids. The decrease in lipid radioactivity at 60 min is not representative of most results. Usually there is little chase of ^{32}P out of phospholipids over periods of up to 4 hours.

Consequently 45 min and 30 min periods for labeling and chase, respectively, were chosen as standard conditions. These times allow the measurement of the rate of phospholipid synthesis without involving excessive time periods during which other unknown effects could take place. A few of the earliest experiments used 30 min for labeling and 15 min for chase. These are noted as they occur.

Figure 2. Short-term chase of aleurone layers. Aleurone layers which had been preincubated for 9 hr in GA were labeled 45 min in 200 μc ^{32}Pi. They were then rinsed and further incubated in 50 mM KH_2PO_4 for the indicated periods at which time the radioactivity in the cellular components was determined.


100
80
60
40
20
0
% of Maximum cpm
Lipid
Organic
Uptake
0
30
60
Time (min)

Determination of Incorporated Radioactivity

Aleurone layers were homogenized in a mortar with sand and a total of 3.5 ml grinding buffer (0.1 M HEPES, pH 7.55 and 0.45 M sucrose). The homogenate was centrifuged at 4,000 x g for 10 min followed by a centrifugation at 10,000 x 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; 1) aliquots were counted directly for total uptake; partitioned to separate 2) ^{32}Pi from 3) phosphate-containing organic compounds; and extracted to yield 4) the phospholipid fraction.

Radioactivity in organic phosphates was assayed by the method of Saha and Good (1970). To 1.0 ml of supernatant was added 5 ml of 10% perchloric acid, 1.0 ml acetone, and 1.0 ml of 10% ammonium molybdate. After stirring, 10 ml of n-butanol-benzene (1:1) was added and the two-phase mixture was again vigorously stirred. At this point 1.0 ml of the upper (butanol-benzene) phase was transferred to a scintillation vial for counting ^{32}Pi. The rest of the upper phase was removed by suction, and the lower phase was filtered through Whatman #4 filter paper premoistened with water. The filter paper retains any small amounts of the upper phase not removed by suction. An additional 0.1 ml of ammonium molybdate and 10 ml of

butanol-benzene were added to the filtrate. After stirring, all traces of the upper phase were removed by suction. A 1.0 ml aliquot of the lower (aqueous) phase was counted. It was experimentally determined that two butanol-benzene extractions gave complete removal of ^{32}Pi from the aqueous phase.

The procedure outlined above results in the partitioning of all inorganic phosphate into the 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 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 by the perchloric acid and are removed by the 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. Likewise, counting the butanol-benzene phase gives an estimate of the uptake and retention of ^{32}Pi in the aleurone layers. It was found that the ratio, ^{32}Pi : uptake, was fairly constant throughout any given experiment (see Table 1). Therefore results are expressed in terms of uptake rather than ^{32}Pi even though both measurements were made. Uptake includes incorporated

Table 1. Ratio of ^{32}Pi to total uptake for a 24 hr time course.

Time (hr)	$\frac{^{32}Pi}{uptake}$ x 100	
	-GA	+GA
4	67	67
8	67	53
12	71	54
18	72	72
24	63	66

Aleurone layers incubated with or without GA were pulse-labeled with ^{32}Pi at the indicated times and radioactivity determinations were made.

^{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 essentially following the procedures of Folch, et al. (1957). Usually 1 ml of supernatant was vigorously extracted with 4-5 ml of chloroform-methanol (2:1). 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) 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 3 washes were sufficient to break emulsions in the chloroform phase and to remove all non-phospholipid ^{32}P trapped in that manner.

Pellets were first extracted with chloroform:methanol (1:1) and centrifuged to remove all debris and precipitated protein. Then sufficient volumes of chloroform and water (containing 0.8% NaCl and 0.2% $MgCl_2$) were added to give a final ratio of chloroform:methanol:water of 8:4:3 which is needed for best phase separation. After mixing followed by removal of the upper phase, the lower phase was washed as described above. The chloroform phases were either concentrated for thin-layer chromatography or transferred to a scintillation vial and dried for counting.

Thin-Layer Chromatography

Phospholipids were separated by thin-layer chromatography (TLC) for estimation of radioactivity in individual compounds. Pre-coated 20 cm x 20 cm TLC glass plates with a 0.25 mm silica gel layer (E. Merck, Darmstadt, Germany) were purchased from Brinkmann Instruments, Inc.

After activation at 105°C for 20 min, the plates were allowed to cool, and samples were spotted in chloroform: methanol (4:1). The plates were developed in a 2-dimensional system of Rouser, et al. (1967). In the first direction chloroform-methanol-14 N NH_4OH (65:35:5) was used. After drying, the second dimension was developed in chloroform-acetone-methanol-acetic acid-water (100:40: 20:20:10).

Visualization was accomplished first with iodine vapor and then by several specific sprays: the Dragendorf reagent for choline-containing phospholipids (Block, et al., 1958), the ninhydrin spray for phosphatidyl ethanolamine and phosphatidyl serine; and the molybdenum blue reagent of Dittmer and Lester for all phospholipids (both summarized by Skipski and Barclay, 1969). 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 was carried out using the method of Bartlett (1959). Suitable aliquots were digested in H_2SO_4, and the color was developed with ammonium molybdate and Fiske-SubbaRow reagent. Appropriate amounts of an orthophosphate standard were also carried through the procedure for conversion of optical density to μmoles Pi.

Measurement of Radioactivity

The scintillation fluid used for all measurements was a mixture of toluene-Triton X-100 (2:1) which contained 4g PPO and 0.1g POPOP per liter of toluene (Patterson and Greene, 1965). This mixture has the advantage of being able to solubilize phospholipids as well as adequately emulsify aqueous samples. No corrections for quenching were needed when 1.0 ml acidic aqueous ^{32}P samples were counted in 20 ml of scintillation fluid.

RESULTS

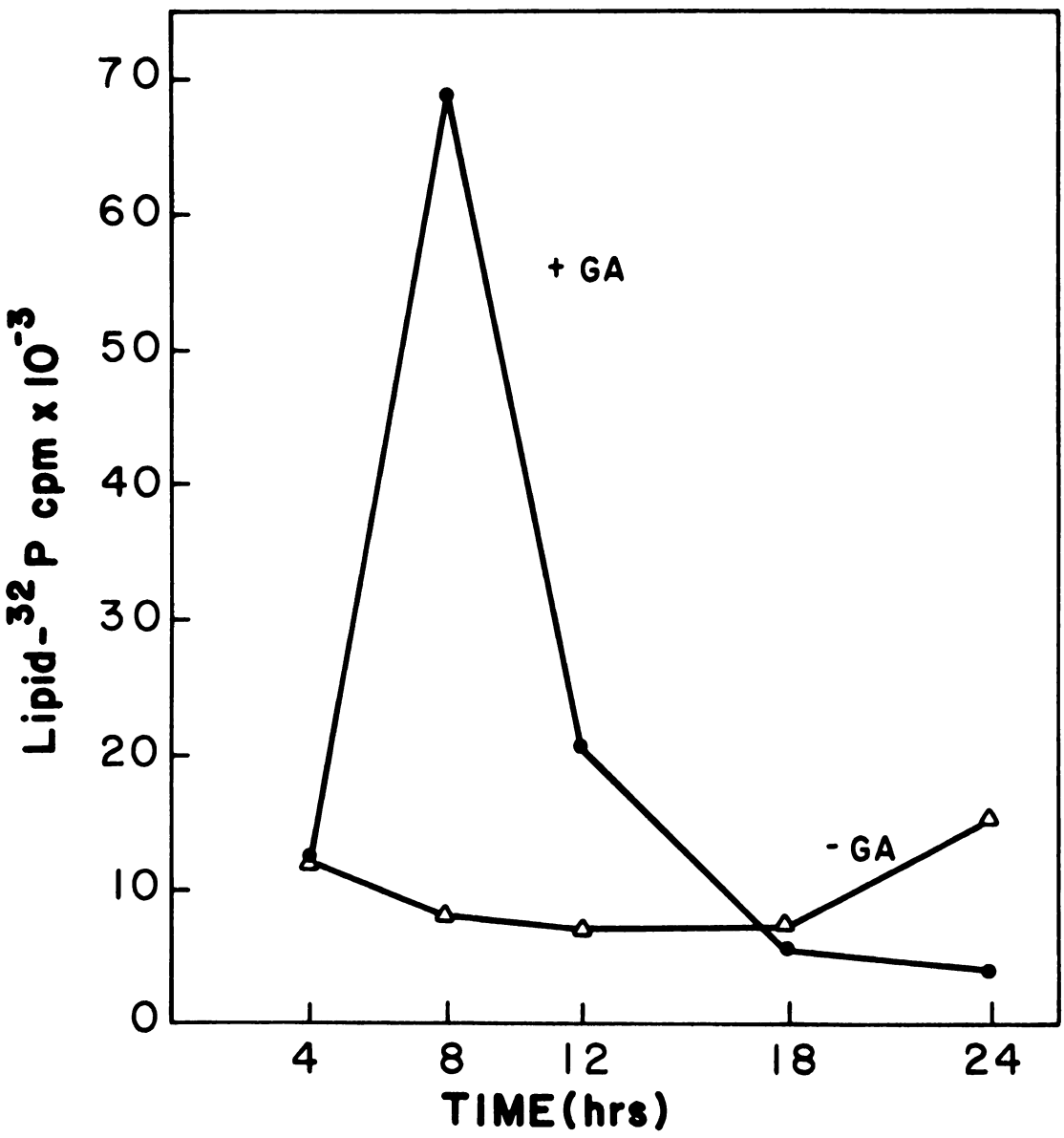
Time Course of Phospholipid Synthesis

Figure 3 shows a typical time course of the rate of phospholipid synthesis in the presence and absence of GA. Phospholipids were extracted from the 10,000 x g supernatant of an aleurone layer homogenate after pulse labeling at the indicated times as described in Materials and Methods. After 4 hours the rate of incorporation of ^{32}Pi into phospholipids increases rapidly, reaching a maximum 8-12 hours after the addition of GA. The rate of incorporation then decreases and reaches the level of the -GA control by 18-24 hours.

Throughout the first 24 hours of incubation, there is a basal level of phospholipid synthesis in the -GA control tissue which remains relatively unchanged. By 24 hours there may be a slight increase in phospholipid synthesis in the control aleurone layers.

In order to follow routinely the uptake and metabolism of ^{32}Pi, two additional measurements were made in all experiments. Total uptake was monitored by counting an aliquot of the 10,000 x g supernatant. Incorporation of

Figure 3. Time course of the rate of ^{32}Pi incorporation into phospholipids. Aleurone layers were pulse labeled at the indicated times and ^{32}Pi incorporation into the phospholipids of the 10,000 x g supernatant was measured.

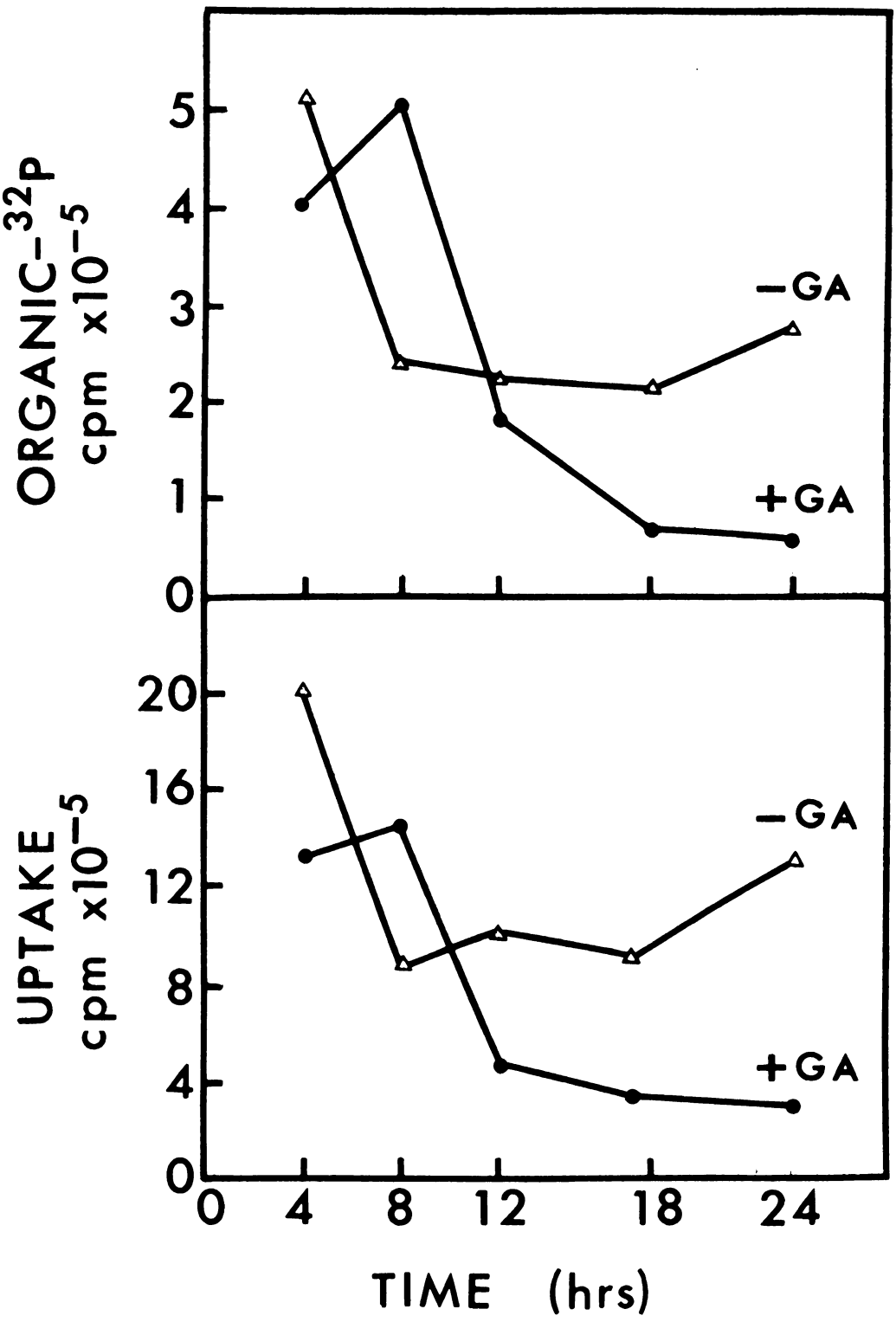

70
60
50
40
30
20
10
0
Lipid-^{32}P cpm x 10^{-3}
+ GA
- GA
4
8
12
18
24
TIME(hrs)

^{32}Pi into organic phosphates was measured as described in Materials and Methods. In this way, effects on ^{32}Pi uptake and metabolism by any of the various treatments can be detected and corrected for when estimating the actual rates of phospholipid synthesis.

In Figure 4 it is shown that ^{32}Pi uptake does vary during the 24 hour time course in both control and GA-treated aleurone layers. 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 fluctuations in uptake are directly reflected in the incorporation of ^{32}Pi into phosphate-containing organic compounds (Figure 4). In addition, the relative rates of ^{32}Pi incorporation into phospholipids are probably being affected in a similar manner. Therefore, it was decided that a more accurate measurement of phospholipid synthesis 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. An increase in the ratio of phospholipid radioactivity to organic phosphate radioactivity indicates a true increase in the rate of phospholipid

Figure 4. Time course of ^{32}Pi uptake into aleurone layers and incorporation of ^{32}Pi into phosphate-containing organic compounds.


ORGANIC-^{32}P
cpm x10^{-5}
5
4
3
2
1
0
−GA
+GA
UPTAKE
cpm x10^{-5}
20
16
12
8
4
0
−GA
+GA
0
4
8
12
18
24
TIME (hrs)

synthesis, 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 (Figure 5), the time course is only slightly different from that of Figure 3. The rate of phospholipid synthesis in GA-treated layers still has a maximum at 8 hours, but the decrease is more gradual and approaches the level of the control layers only after 24 hours.

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

The above arguments also suggest the calculation of the ratio of organic-^{32}P to uptake of ^{32}Pi. This ratio will show if there are different rates of incorporation of ^{32}Pi into the organic phosphate pools for a given amount of ^{32}Pi uptake. Figure 6 shows that in GA-treated layers the ratio, organic-^{32}P:^{32}Pi uptake, is increased over the -GA control ratio by 29% and 72% at 8 and 12 hours, respectively. This result shows there is a more rapid metabolism and incorporation of ^{32}Pi into the organic phosphate fraction of aleurone layers incubated with GA. By 18 hours the effect is no longer visible.

Figure 5. Time course of phospholipid synthesis. Incorporation of ^{32}Pi into phospholipids is expressed as a percentage of the ^{32}Pi incorporation into organic phosphates.

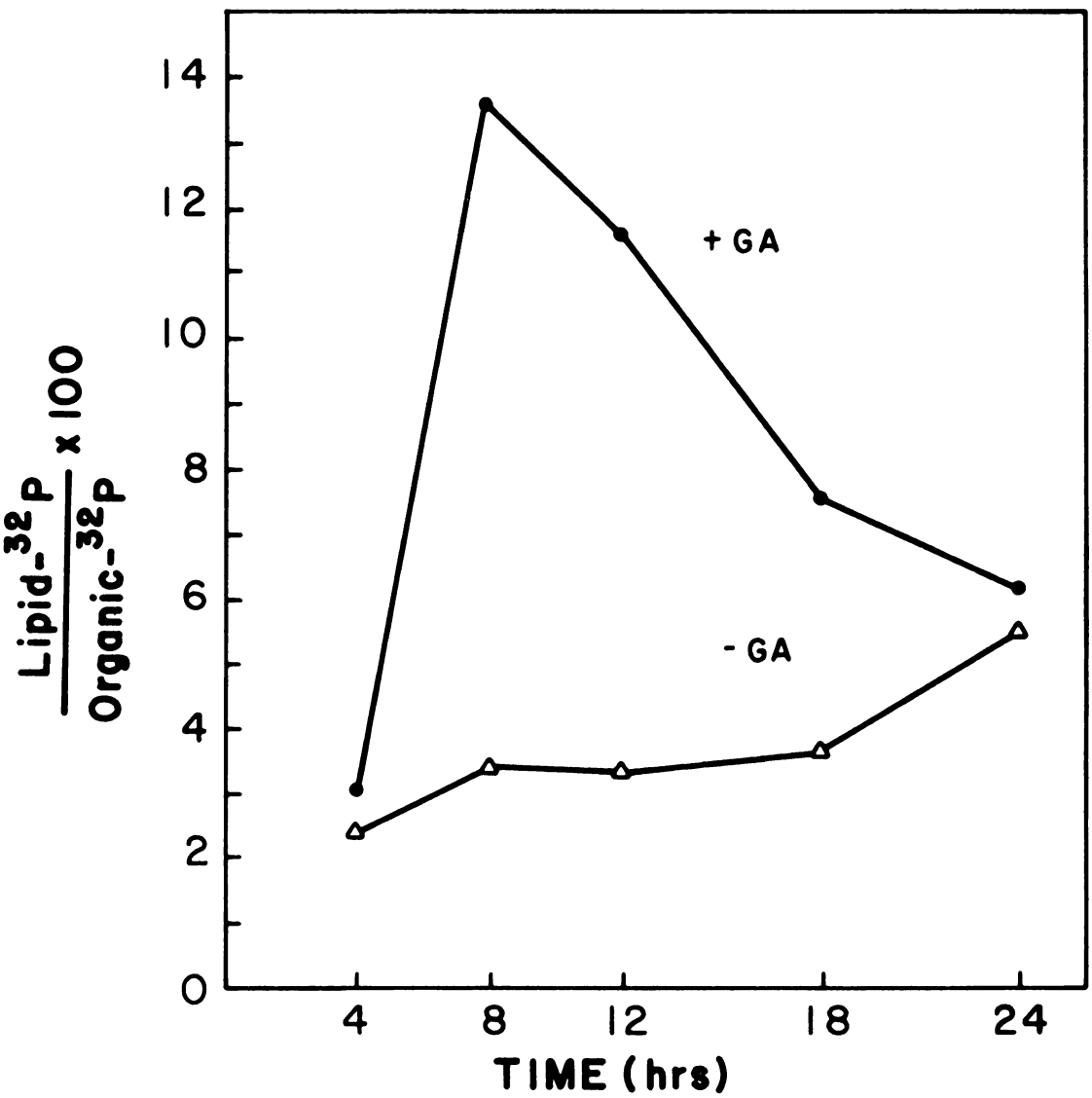
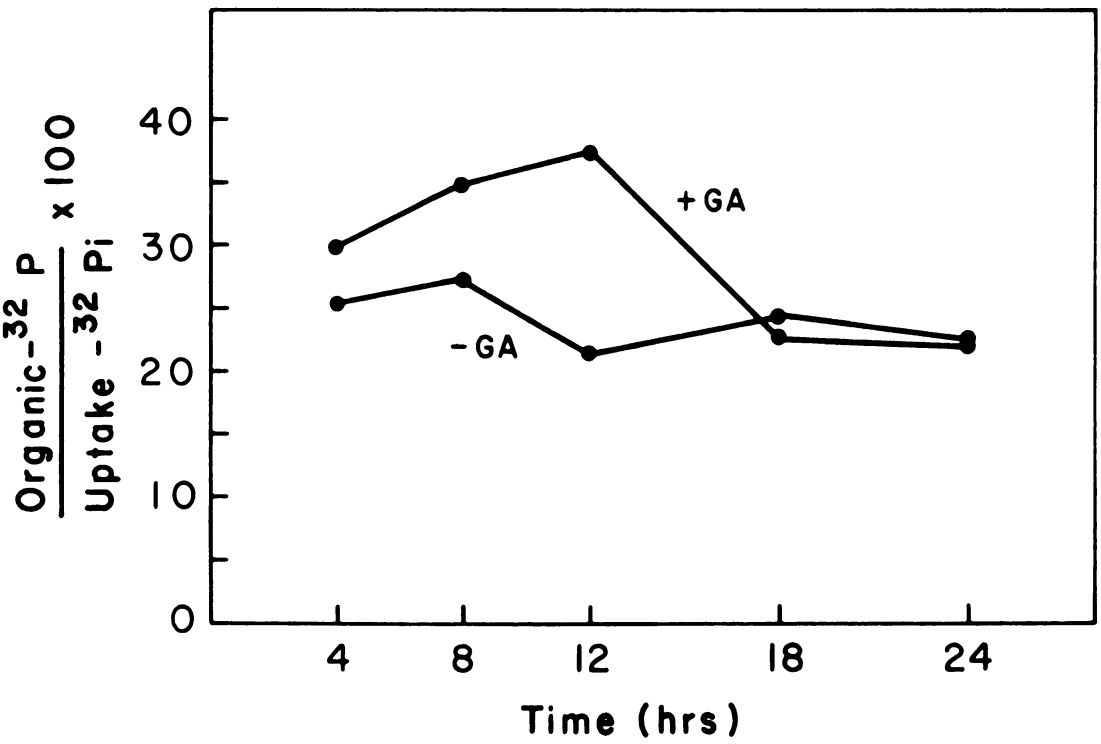

14
12
10
8
6
4
2
0
+ GA
- GA
4
8
12
18
24
TIME (hrs)
Lipid-32P / Organic-32P x 100

Figure 6. Ratio of ^{32}Pi incorporation into organic phosphates to uptake of ^{32}Pi for the 24 hr time course.


Organic-32 P / Uptake -32 Pi x 100
40
30
20
10
0
+GA
-GA
4
8
12
18
24
Time (hrs)

In summary, the time course of the rate of phospholipid synthesis in response to GA has been measured and shows a maximum after 8-12 hr of hormone treatment. The most satisfactory method of expressing the results is to calculate the ratio of phospholipid-^{32}P : organic-^{32}P. Uptake of ^{32}Pi is also monitored and the ratio of organic-^{32}P to uptake also shows enhancement by GA.

Characterization of ^{32}Pi Incorporation

In order to be sure that the GA enhancement of phospholipid synthesis 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 x g and 10,000 x g pellets as well as the supernatant from plus and minus GA treatments were extracted with chloroform : methanol and the phospholipids were counted. Table 2 shows that the GA enhancement of ^{32}Pi incorporation into phospholipids occurs in all three cell fractions. The 4,000 x g pellet which contains unbroken cells, cell debris, and nuclei, the 10,000 x g pellet which should be 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.

Table 2. Distribution of labeled phospholipids in different cell fractions

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

Twenty aleurone layers per flask were incubated for 8 hours with or without 1 μM GA_3. At that time they were labeled with 225 μc ^{32}Pi per flask for 30 min and chased with 0.05 m KH_2PO_4 an additional 15 min.

These 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. In addition these data provide no evidence that synthesis of endoplasmic reticulum is preferentially enhanced. Although purified membrane fractions were not examined, the GA enhancement was the same in all fractions obtained, including the presumably ER-rich supernatant.

A second experiment demonstrates the particulate nature of the phospholipids from the 10,000 x g supernatant which are routinely analyzed for most experiments. It is shown in Table 3 that 80-90% of the phospholipids in the 10,000 x g supernatant can be pelleted at 105,000 x g for 1 hr. If a 105,000 x g pellet is treated with 0.1% Triton X-100, 95% of the radioactivity is solubilized and is not repelleted during a second centrifugation at 105,000 x g. Therefore, these characteristics of pelletability and detergent solubility indicate that the chloroform : methanol soluble radioactivity of the 10,000 x g supernatant is associated with a structure that behaves like a membrane and can be attributed to phospholipid.

Final confirmation of the assumption that chloroform : methanol soluble ^{32}P-radioactivity from various cell fractions actually represents phospholipids comes from thin-layer chromatography (TLC) of these chloroform : methanol

Table 3. Particulate nature of supernatant phospholipids.

Fraction extracted	-GA	+GA
	cpm	cpm
10,000 x g supernatant	1,400	17,000
105,000 x g pellet	1,300	16,000
105,000 x g supernatant	200	2,400
105,000 x g pellet treated with detergent and repelleted at 105,000 x g	50	540
Supernatant of detergent-treated pellet	900	10,600

Portions of the 10,000 x g supernatant from layers incubated for 8 hrs with or without GA were extracted with chloroform : methanol or were centrifuged at 105,000 x g for 1 hr followed by chloroform : methanol extraction of the pellet and supernatant. Additional 105,000 x g pellets were suspended in 0.1% Triton X-100 and centrifuged a second time at 105,000 x g for 1 hr. The supernatant and pellet obtained from this centrifugation were then extracted with chloroform : methanol.

extracts. Samples were spotted on 20 x 20 cm pre-coated silica gel plates and developed in two dimensions. Sprays specific for phosphates, amino groups, and choline as well as chromatography of authentic standards gave positive identification of most of the phospholipids. Figure 7 shows a representative separation of the phospholipids in a chloroform-methanol extract of barley aleurone layers. The following phospholipids were detected chemically and by the presence of radioactivity:

Phosphatidyl choline

Phosphatidyl ethanolamine

Phosphatidyl inositol

Phosphatidyl glycerol

Cardiolipin (tentative)

Phosphatidic acid

The GA-enhanced increase in radioactivity seemed to be distributed among most of the labeled phospholipids (Table 4). Small increases in the relative amounts of radioactivity in phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl inositol at the expense of phosphatidyl glycerol may have occurred at this time. 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 general proportionate increase in labeling for all phospholipids (except phosphatidyl glycerol).

Figure 7. Schematic diagram of the separation of phospholipids by TLC. Chloroform-methanol extracts of aleurone layers were concentrated and applied to activated, precoated TLC plates at the origin, O. Solvent systems used for development were: (1) chloroform-methanol-14N ammonia (65:35:5); and (2) chloroform-acetone-methanol-acetic acid-water (100:40:20:20:10). The following phospholipids were identified: PC, phosphatidyl choline; PE, phosphatidyl ethanol amine; PI, phosphatidyl inositol; PG, phosphatidyl glycerol; PA, phosphatidic acid. The identification of cardiolipin (C) is tentative. In some samples an additional phosphorus-containing compound was detected (X). The approximate locations of phosphatidyl serine (PS) and lysophosphatidyl choline (LPC) are indicated but were barely detectable chemically, and no radioactivity was associated with these areas. No radioactivity remained at the origin.

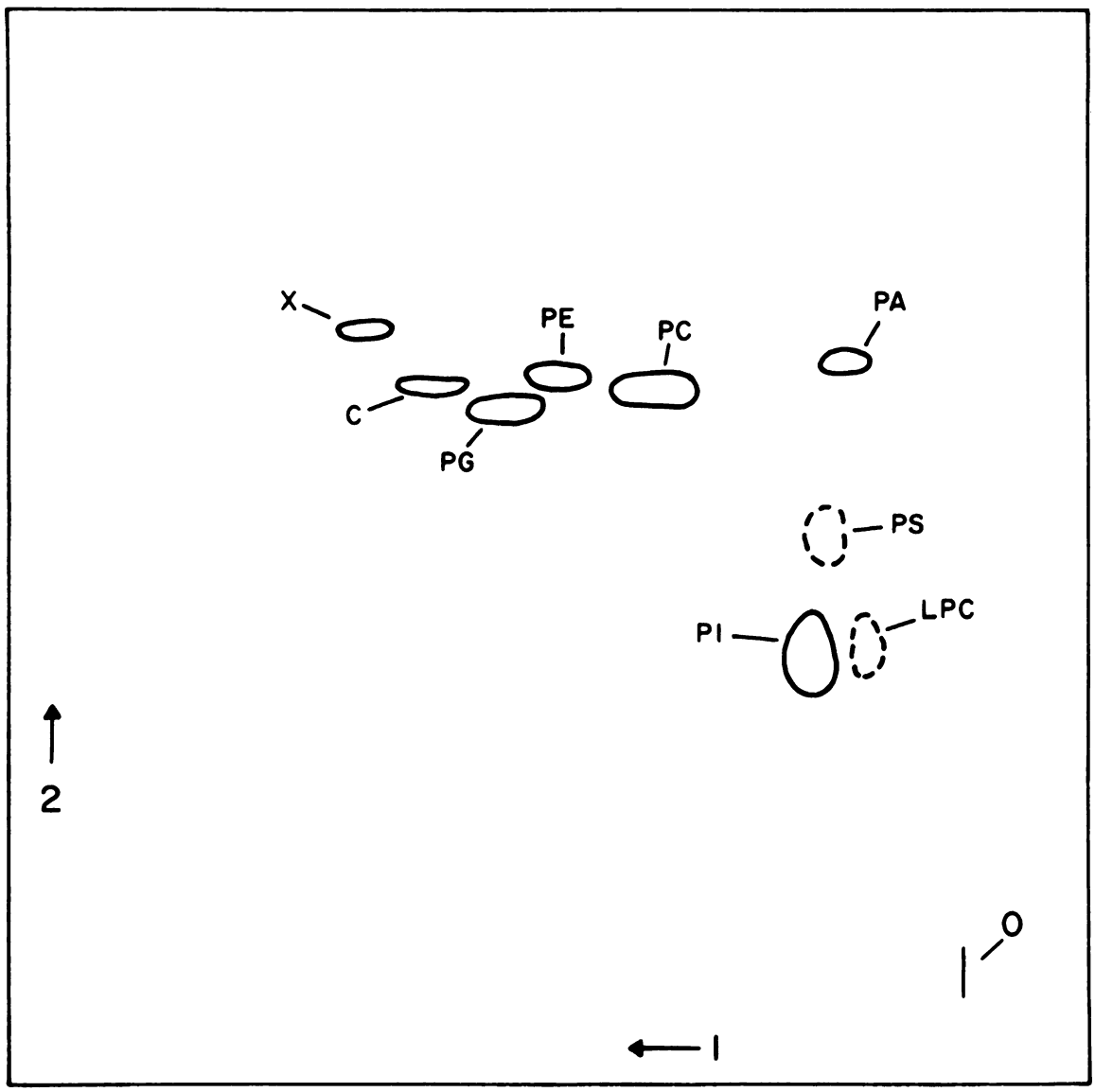

X
PE
PC
PA
C
PG
PS
PI
LPC
2
O
1

Table 4. Relative distribution of radioactivity in individual phospholipids.

	Per Cent of Incorporated Radioactivity in Each Compound	
	-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

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 thin-layer chromatography of the washed extracts. Approximately 3,000 cpm and 25,000 cpm were analyzed for -GA and +GA samples, respectively. The above compounds account for 97% and 92%, respectively, of the radioactivity which was analyzed.

The characterization experiments, then, suggest that the GA enhancement of ^{32}Pi incorporation into phospholipids is a rather general phenomenon in the cell. The stimulation does not seem to be localized in any one cell fraction or confined to a particular phospholipid, but is found in all cell fractions and all labeled phospholipids.

Effects of Metabolic Inhibitors

The GA-induced enhancement of alpha amylase synthesis is inhibited to varying degrees by several metabolic inhibitors (Chrispeels and Varner, 1967a, 1967b). Cycloheximide completely inhibits amylase synthesis almost immediately while the inhibition by actinomycin D is less severe.

Figure 8 shows that phospholipid synthesis is also rapidly inhibited by cycloheximide. One hour after the addition of cycloheximide, the rate of phospholipid synthesis 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 phospholipid synthesis but does not affect the basal rate of phospholipid synthesis in -GA layers (Table 5). However, cycloheximide added at 0 time reduces phospholipid synthesis in control and +GA layers to values below basal levels.

Figure 8. Time course of inhibition of phospholipid synthesis 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 and phospholipids were extracted.

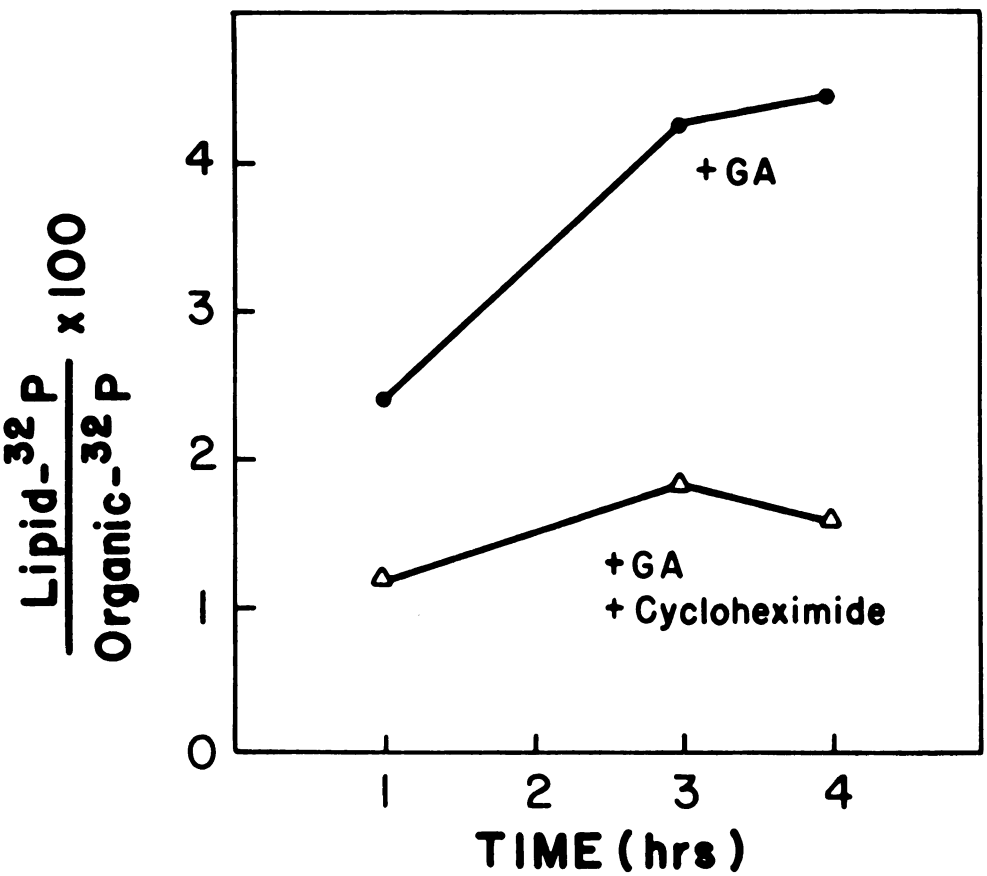

Lipid-32P / Organic-32P x100
0
1
2
3
4
1
2
3
4
TIME (hrs)
+GA
+GA
+ Cycloheximide

Table 5. Cycloheximide inhibition of phospholipid synthesis in control and GA treated aleurone layers.

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

Aleurone layers were incubated as described above. All treatments were labeled after 7 hr of incubation. Phospholipids were extracted and counted.

Therefore these experiments indicate that there is a requirement for continued protein synthesis in order for a GA enhancement of phospholipid synthesis to take place. Cycloheximide specifically inhibits hormone-induced increases in the rate of phospholipid synthesis within 1 hr but has no effect on the rate in control layers for short time periods.

On the other hand, the possibility does exist that the inhibition of phospholipid synthesis by cycloheximide is due to other indirect effects of the drug rather than a direct inhibition of protein synthesis.

In a similar experiment, 6-methylpurine was also shown to inhibit the GA enhancement of phospholipid synthesis. Barley half seeds were incubated in GA for 7 hours. Aleurone layers were then separated from the endosperm and again incubated in GA with or without 6-methylpurine. At 0, 2, and 4 hours sets of layers were labeled with ^{32}Pi and phospholipids were extracted and counted. While the rate of phospholipid synthesis continues to increase in layers treated with GA only, 6-methylpurine has inhibited most of this increase after 2 hours, and after 4 hours the inhibition is even greater (Figure 9). Amylase synthesis is effectively inhibited by 6-methylpurine, probably because of an inhibition of RNA synthesis (Chrispeels and Varner, 1967b). Phospholipid synthesis may therefore be indirectly inhibited via the inhibition of RNA synthesis by 6-methylpurine.

Another inhibitor of RNA synthesis, actinomycin D, also inhibits phospholipid synthesis (Table 6). When actinomycin

Figure 9. Inhibition of phospholipid synthesis 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 hours layers were pulsed 30 min with ^{32}Pi followed by a 15 min chase and phospholipid extraction.

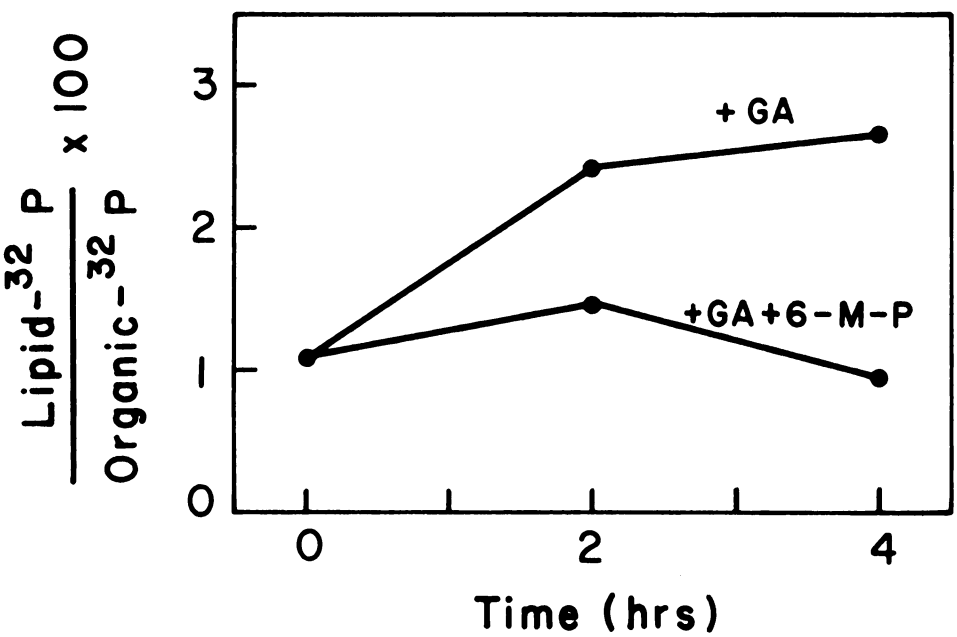

Lipid-32 P / Organic-32 P x 100
3
2
1
0
+ GA
+GA+6-M-P
0
2
4
Time (hrs)

Table 6. Effect of actinomycin D on phospholipid synthesis.

Treatment	$\frac{\text{Lipid-}^{32}P}{\text{Organic-}^{32}P} \times 100$
-GA	1.7
+GA	6.4
+GA	
+ act. D (25 µg/ml)	8.4
+ act. D (50 µg/ml)	4.4
+ act. D (100 µg/ml)	3.3

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

D is added at the same time as GA, concentrations of 100 μg/ml and 50 μg/ml inhibit phospholipid synthesis, but there may be a slight stimulation at 25 μg/ml. The extent of inhibition is approximately the same as was found for amylase production when measured after 24 hr (Chrispeels and Varner, 1967a).

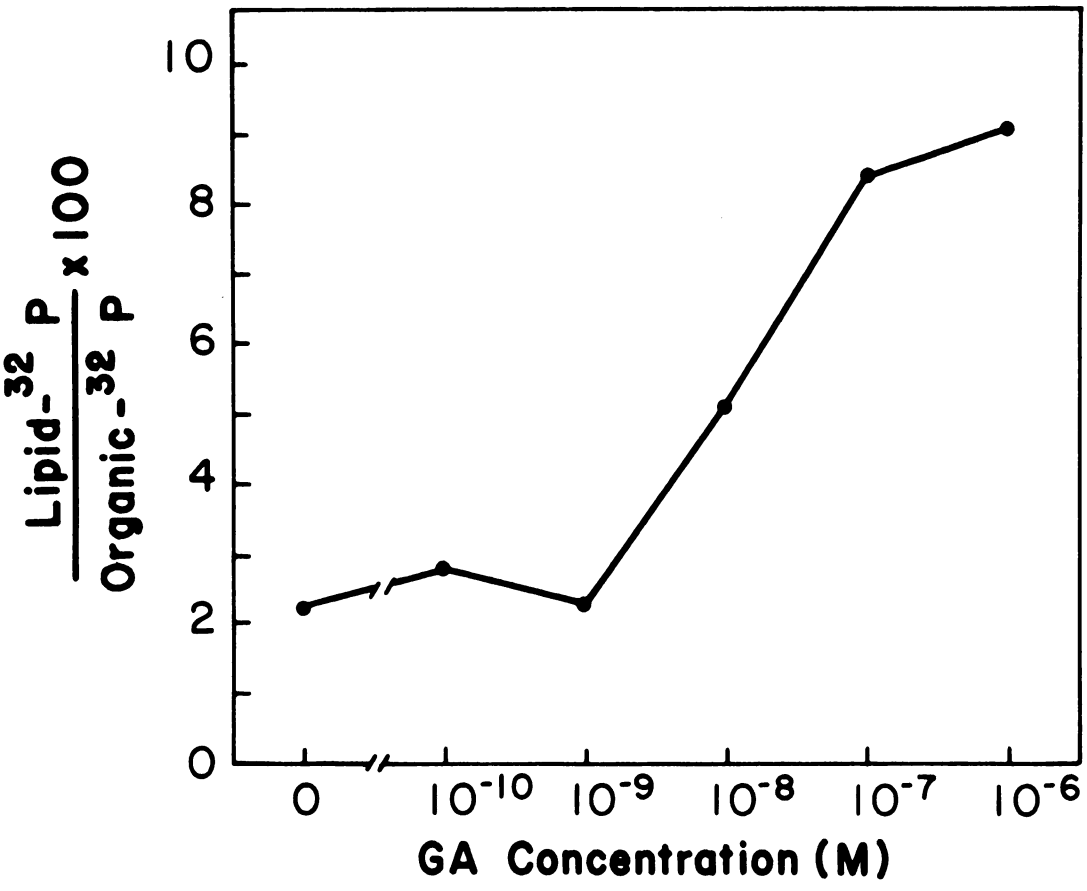
The effects of RNA synthesis inhibitors on phospholipid synthesis correspond to the effects on amylase synthesis, i.e., 6-methylpurine is a more effective inhibitor than actinomycin D and is effective when added during the mid-course of GA action as well. These results indicate a requirement for RNA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

Hormonal Control

The enhancement by GA of the rate of phospholipid synthesis was earlier presented as a time course of the effect. Now additional features of GA control as well as the abscisic acid inhibition of the enhancement will be shown.

The response of phospholipid synthesis to increasing concentrations of GA is shown in Figure 10. Above 10^{-9} M phospholipid synthesis increases with an increase in the GA concentration. A maximum is reached at 10^{-6} M. Although the response is not proportional over quite as wide a range

Figure 10. The response of phospholipid synthesis 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.


Lipid-32 P / Organic-32 P x100
10
8
6
4
2
0
0
10-10
10-9
10-8
10-7
10-6
GA Concentration (M)

of GA concentrations as is α-amylase release (Jones and Varner, 1967), there is a clear dependence of the rate of phospholipid synthesis on GA concentration.

The removal of GA from layers already partially induced results in a decrease in amylase production (Chrispeels and Varner, 1967b) and in the percentage of GA-induced polyribosomes (Evins and Varner, 1972). When the rate of phospholipid synthesis is measured in aleurone layers which have been washed for 3 hrs to remove GA, there is a decrease in the rate compared to the rate in +GA control layers (Figure 11). Layers in GA continuously show a maximum at 10 hr and then decline as expected. Layers subjected to several rinses in -GA medium after a 7 hr incubation in GA show a steady decline in phospholipid synthesis after hour 7. Removal of GA from the layers stops any further increase in the rate of phospholipid synthesis.

It should be pointed out that washing GA out of the layers causes a rather large increase in uptake of ^{32}Pi (Figure 12). Organic-^{32}P shows a similar fluctuation. However, since radioactivity incorporated into phospholipids does not exhibit a proportionate increase, the ratio of phospholipid-^{32}P to organic-^{32}P shows a gradual decline, even though the actual radioactivity in phospholipids is higher in washed layers.

Abscisic acid (ABA) inhibits the GA-induced production of α-amylase in barley aleurone layers (Chrispeels and

Figure 11. Effect of removal of GA on the rate of phospholipid synthesis. Layers incubated in GA for 7 hr were rinsed and transferred to -GA medium at hour 7. The layers were rinsed and transferred to fresh medium every half hour for the first 2 hr and every hour after that. Layers in +GA medium were also rinsed in +GA medium and transferred to new +GA medium. At the indicated times layers were pulse-labeled with ^{32}Pi and phospholipids extracted.

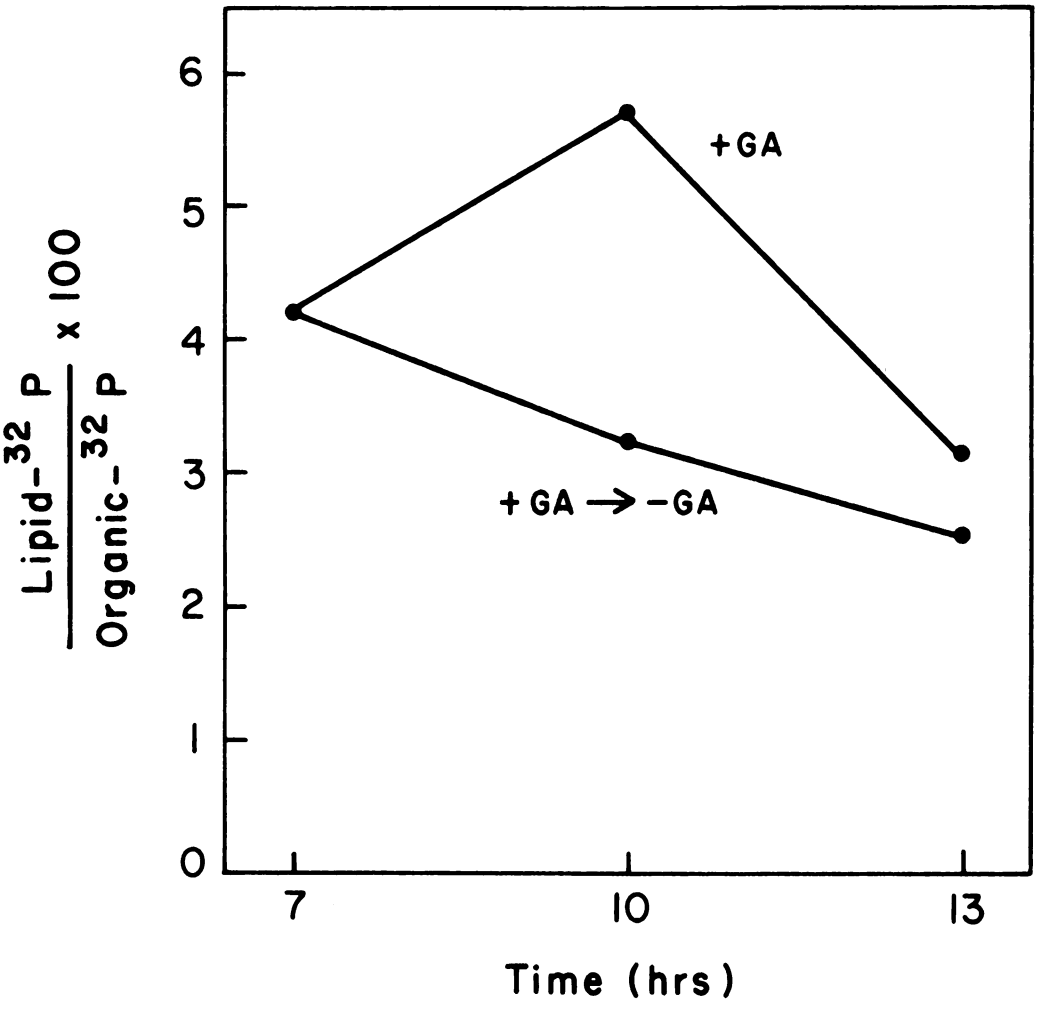
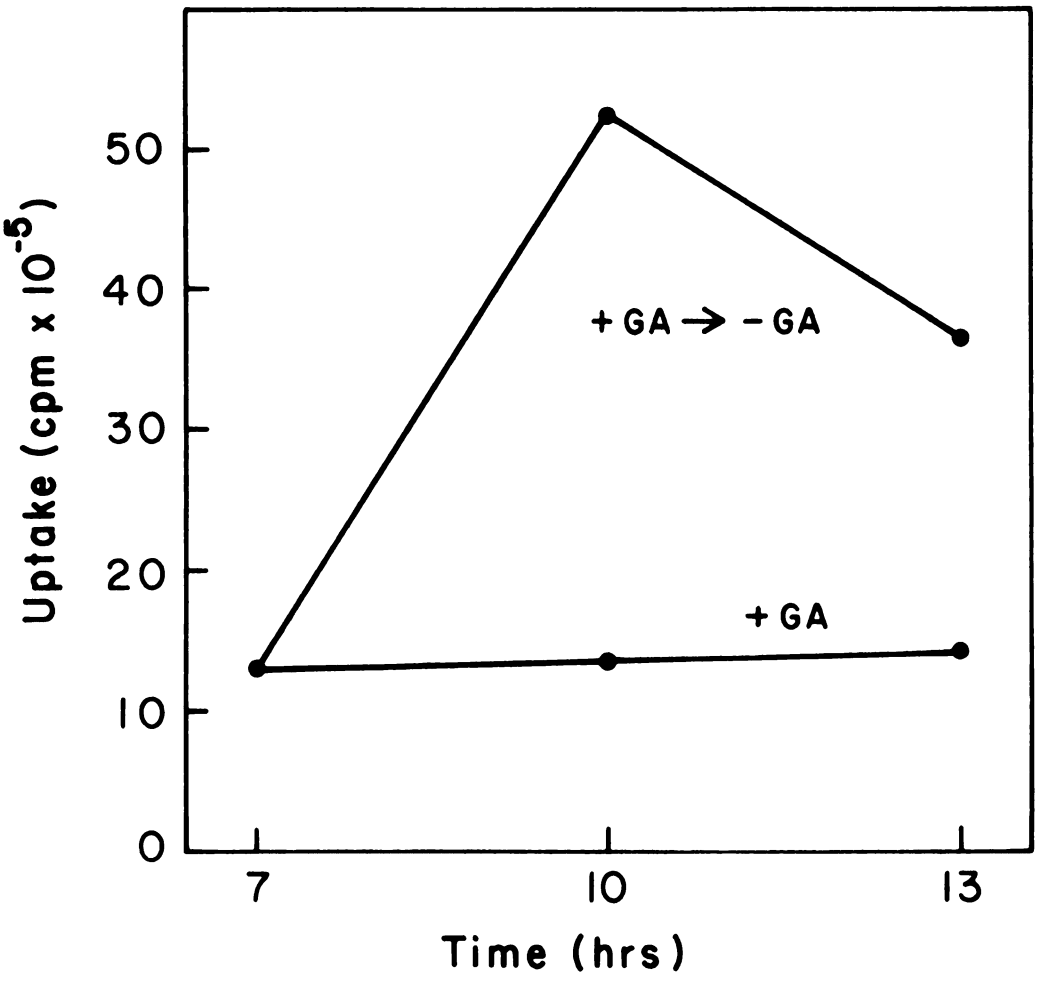

$\frac{\text{Lipid-}^{32}\text{P}}{\text{Organic-}^{32}\text{P}} \times 100$
6
5
4
3
2
1
0
+GA
+GA → -GA
7
10
13
Time (hrs)

Figure 12. Uptake of ^{32}Pi by aleurone layers undergoing the removal of GA. The conditions are the same as for Figure 11.


Uptake (cpm x 10^{-5})
50
40
30
20
10
0
7
10
13
Time (hrs)
+ GA → - GA
+ GA

Varner, 1967b). It will also inhibit phospholipid synthesis. When ABA is added to GA-treated layers, any further enhancement of the rate of phospholipid synthesis is prevented (Figure 13). Other experiments show no effect of ABA on the basal rate of phospholipid synthesis in -GA control layers, whether the ABA is added in mid-course or at 0 time.

The addition of ABA causes an increase in ^{32}Pi uptake in aleurone layers (Figure 14). Increased amounts of radioactivity are then incorporated into organic phosphates and phospholipids. However the rate of phospholipid synthesis is lower in ABA-treated layers because there is proportionately less incorporation into phospholipids of these layers than for +GA layers for a given level of organic phosphate radioactivity.

It is not clear why hormone fluctuations stimulate ^{32}Pi uptake. In the case of ABA it does not always occur. Sudden changes in hormone levels, whether the addition of ABA or the removal of GA, are not representative of physiological conditions. The system may simply be reacting or adjusting to such changes.

The effect of increasing concentrations of ABA on the rate of phospholipid synthesis is shown in Figure 15. There is no inhibition of the GA response until the ABA concentration reaches 0.1 micromolar. At 1.0 μM phospholipid synthesis has been reduced to the level of the -GA layers. The inhibition of phospholipid synthesis is sensitive to the

Figure 13. Inhibition of GA-enhanced phospholipid synthesis by abscisic acid. Forty aleurone layers per flask were incubated in 0.1 μM GA. After 8 hours, 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 extracted.

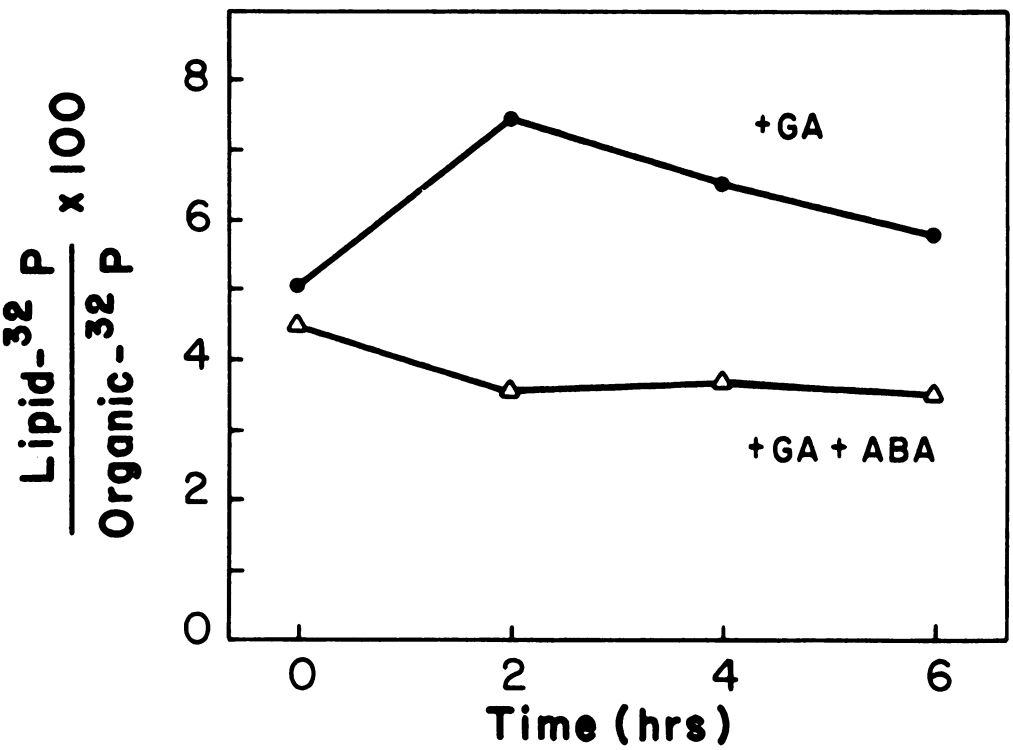

Lipid-32 P / Organic-32 P x 100
8
6
4
2
0
+GA
+GA + ABA
0
2
4
6
Time (hrs)

Figure 14. Effect of ABA on ^{32}Pi incorporation into cellular components. Conditions are as in Figure 13. ABA was added at hour 8.

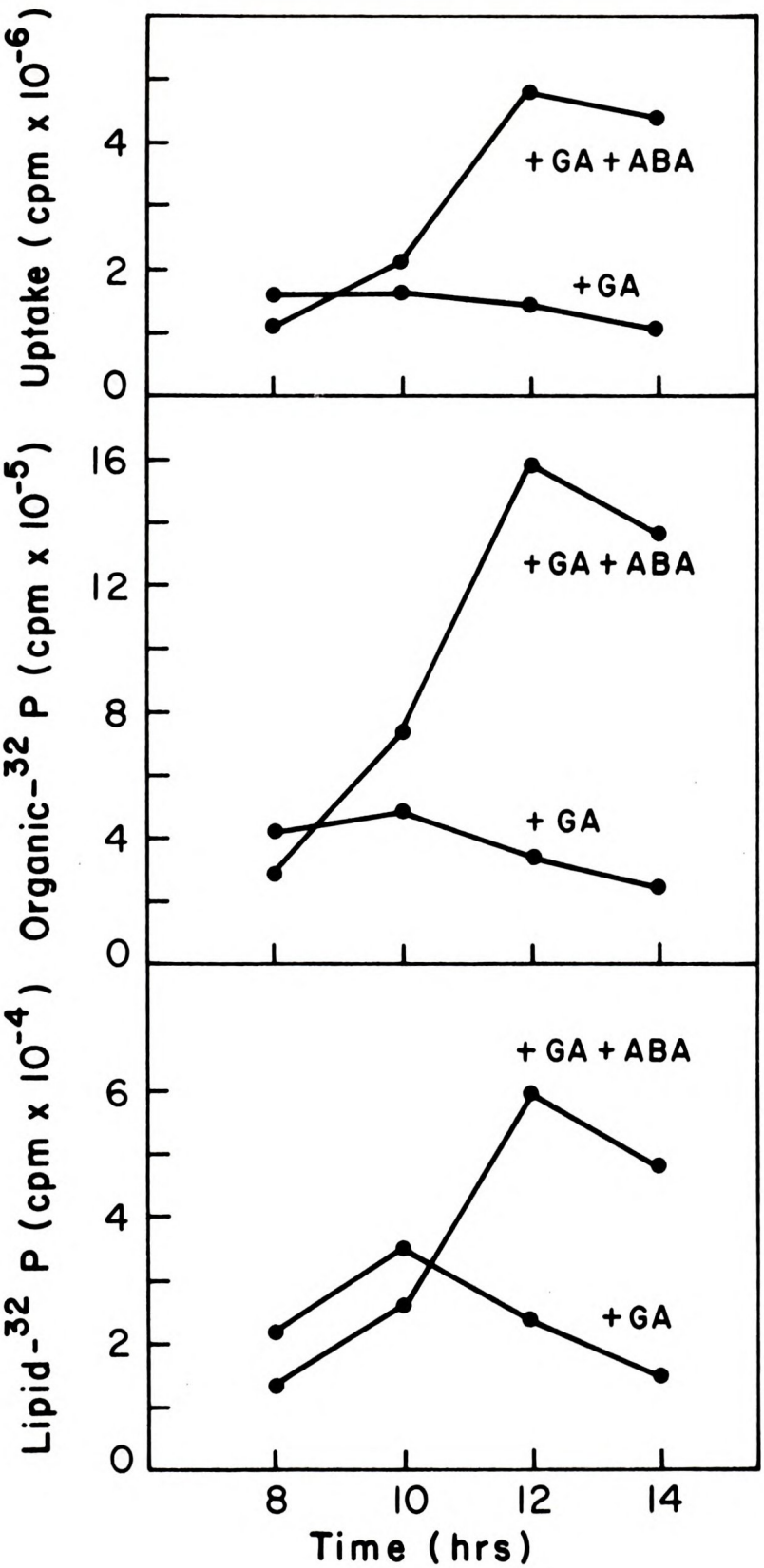
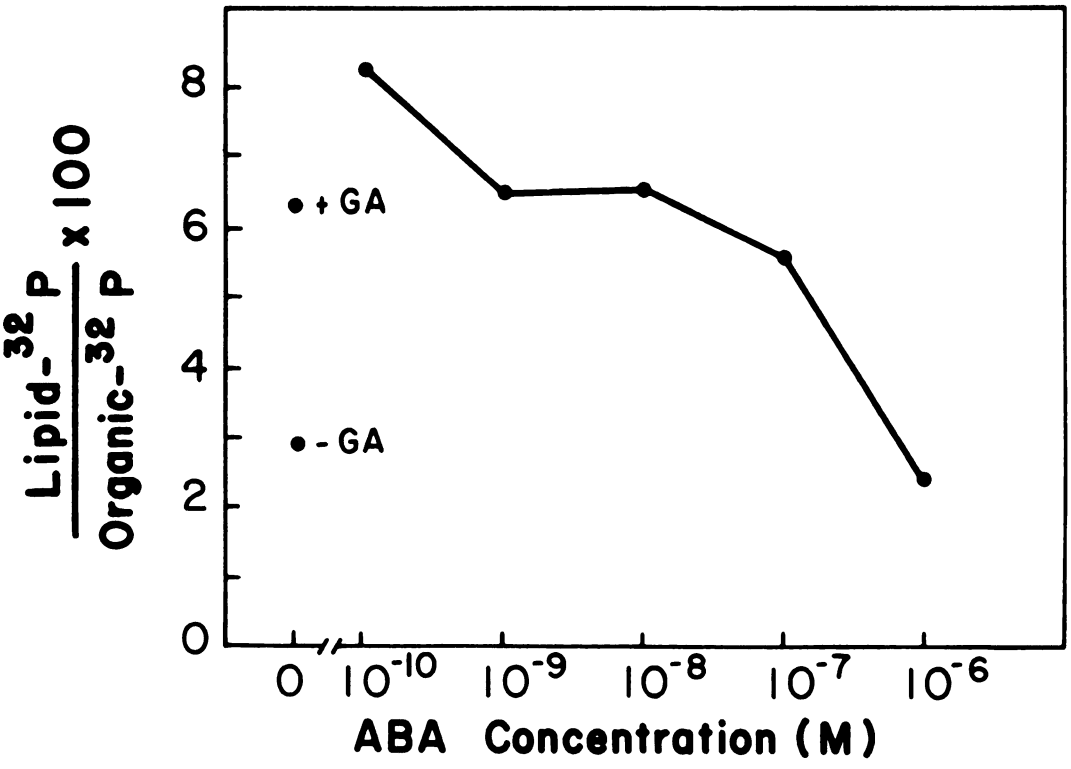

Uptake (cpm x 10^{-6})
Organic-^{32}P (cpm x 10^{-5})
Lipid-^{32}P (cpm x 10^{-4})
+GA + ABA
+GA
+GA + ABA
+GA
+GA + ABA
+GA
0
2
4
0
4
8
12
16
0
2
4
6
8
10
12
14
Time (hrs)

Figure 15. Progressive inhibition of phospholipid synthesis by increasing concentrations of ABA. Aleurone layers were incubated for 8 hours with both GA (1.0 μM) and ABA at the indicated concentrations present from the start. The layers were then pulse-labeled with ^{32}Pi and phospholipids extracted.


$\frac{\text{Lipid-}^{32}P}{\text{Organic-}^{32}P} \times 100$
8
6
4
2
0
+ GA
- GA
0
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
ABA Concentration (M)

concentration of ABA over about two orders of magnitude when the ABA is added at 0 time.

In summary, the evidence for hormonal control of phospholipid synthesis consists of the concentration dependence of responses to GA and ABA as well as the time course of inhibition by ABA. This inhibition by ABA is rapid and is specific for GA-enhanced phospholipid synthesis. Removal of GA also brings about a decline in the rate of phospholipid synthesis.

Other Possible Points of Control

Several other approaches to the problem of the control of phospholipid synthesis have also been examined.

The first involves the observations of Jones (1969a) that osmotic agents such as mannitol or polyethylene glycol inhibit the induction of α-amylase by GA in barley aleurone layers. Figure 16 shows that mannitol also inhibits the rate of phospholipid synthesis. When layers are incubated in GA with increasing concentrations of mannitol, phospholipid synthesis is progressively inhibited until finally it is reduced to the level of the -GA layers. There is no inhibition by mannitol of the basal rate of phospholipid synthesis in -GA control layers.

An additional observation is that the enhancement by GA of the organic phosphate-^{32}P : uptake ratio is not inhibited by mannitol (Table 7). This ratio usually shows

Figure 16. The effect of mannitol on GA-enhanced phospholipid synthesis. Aleurone layers were incubated 8 1/2 hr in the indicated concentrations of mannitol with or without GA. They were then pulse-labeled and phospholipids were extracted.

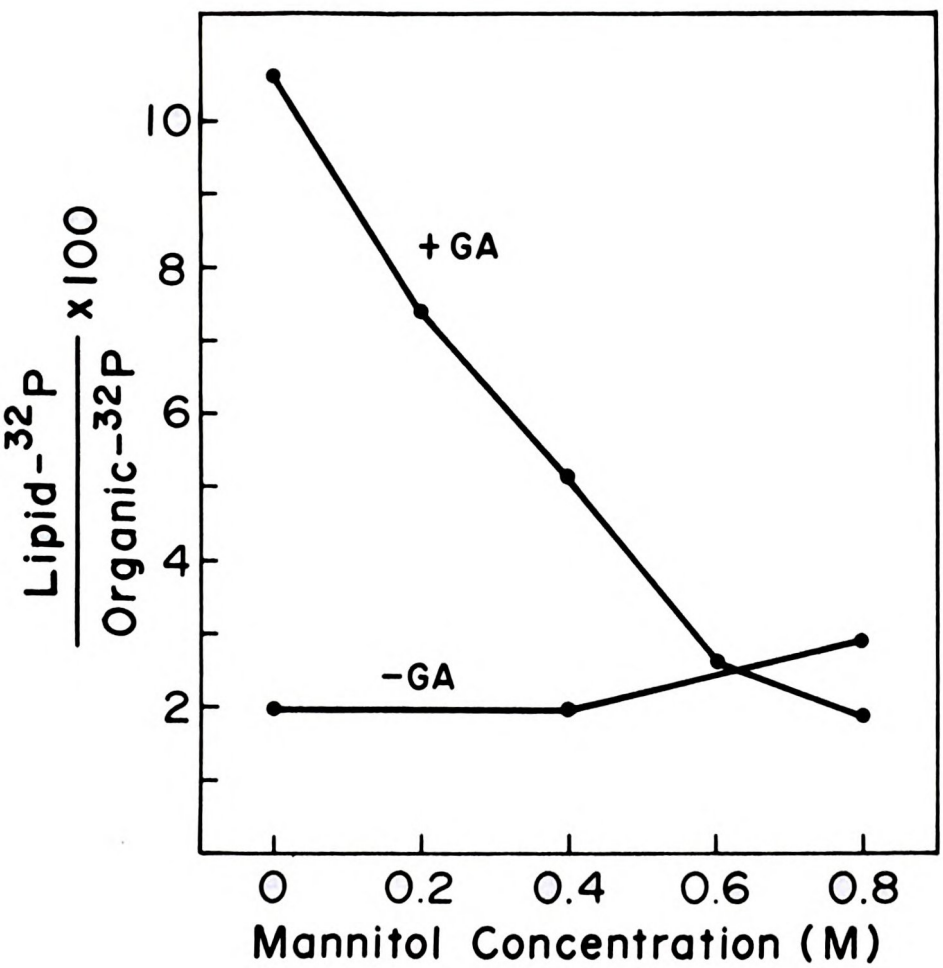

Lipid-32P / Organic-32P x100
10
8
6
4
2
+GA
-GA
0
0.2
0.4
0.6
0.8
Mannitol Concentration (M)

Table 7. Effects of GA and mannitol on the incorporation of ^{32}Pi into organic phosphates.

Mannitol (M)	$\frac{\text{Organic-}^{32}P}{^{32}Pi \text{ Uptake}}$ x 100	
	-GA	+GA
0	10.5	16.0
0.2		16.4
0.4	11.5	22.6
0.6		24.9
0.8	14.1	21.4

Aleurone layers were incubated for 8-1/2 hr in the indicated concentrations of mannitol with or without GA. They were then pulse-labeled and phospholipids were extracted.

at least a 50% increase with GA treatment. While mannitol has completely inhibited GA-enhanced phospholipid synthesis, the stimulation of the ratio of organic-^{32}P to uptake of ^{32}Pi has not been affected. This means mannitol inhibits phospholipid synthesis and α-amylase formation to similar extents, but leaves untouched another effect of GA, namely the enhancement of the organic phosphate : uptake ratio.

Recent work with the barley endosperm system has suggested that 3', 5'-cyclic AMP (cAMP) may mediate the action of (Pollard, 1970) or substitute for GA (Galsky and Lippincott, 1969). Phospholipid synthesis, however, is not stimulated by cAMP nor by N^6, $O^{2'}$-dibutyryl cAMP (Table 8). Although experiments were not performed using a sub-optimal concentration of GA as in some of the cAMP work, there is no enhancement of phospholipid synthesis in the absence of GA. In the presence of a saturating level of GA, cAMP appears to inhibit the rate of phospholipid synthesis. Therefore, if cAMP has a role in the enhancement of α-amylase by GA, there is no indication of such a role in its effects on phospholipid synthesis.

Additional Biochemical Data

In an effort to learn more about the nature of the phospholipid synthesis enhanced by GA, the incorporation of ^{14}C-acetate by aleurone layers was examined. Table 9 shows there was no effect of GA on the incorporation of

Table 8. Effects of 3', 5'-cyclic AMP and N^6, $O^{2'}$-dibutyryl cAMP on the rate of phospholipid synthesis.

Treatment	$\frac{\text{Lipid-}^{32}P}{\text{Organic-}^{32}P} \times 100$	
	-GA	+GA
Control	4.5	11.8
Cyclic AMP	3.5	5.9
Dibutyryl cAMP	4.2	6.0

Aleurone layers were incubated with or without GA (1 μM) for 8 hr at which time they were pulse-labeled and phospholipids were extracted. The cAMP compounds were present from the beginning of the incubation period at a concentration of 5 mM.

Table 9. Incorporation of ^{14}C-acetate by aleurone layers.

	-GA	+GA
	(cpm x 10^{-4}/10 layers)	
Chloroform-methanol soluble	7.1	7.5
TCA insoluble	14.9	14.3
Uptake of ^{14}C-acetate	60.3	73.5

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% TCA and filtered through a nitrocellulose filter.

acetate into compounds soluble in chloroform-methanol or insoluble in TCA. 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 organic phosphate and phospholipid levels in aleurone layers were also carried out. The 4,000 x g and 10,000 x g pellets and the 10,000 x g supernatant were analyzed for phospholipids separately and the values combined to give the total phospholipid content of the layers (Table 10).

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 2). That is, the 4,000 x g pellet, the 10,000 x g pellet, and the 10,000 x g supernatant contained about 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.

The phosphorus level of the organic phosphate fraction obtained from the 10,000 x g supernatant was also determined (Table 11). Here layers incubated in GA show a 50% increase in the chemical level of organic phosphorus at 12 and 18 hours.

Table 10. Phospholipid content of aleurone layers.

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

Phospholipids were extracted from the 4,000 x g and 10,000 x g pellets and supernatant of aleurone layer homgenates. The values above are a sum of these three determinations.

Table 11. Organic phosphate content of aleurone layers.

Time	Organic-P (μmoles/10 layers)	
	-GA	+GA
6	4.6	4.8
12	4.8	7.6
18	3.8	7.0
24	3.8	5.3

Chemical determinations of phosphorus were made on the organic phosphate fraction of the 10,000 x g supernatant of the homogenate of aleurone layers incubated as shown above.

This increase is similar to the GA-enhanced incorporation of ^{32}Pi into organic phosphates of Figure 6, but the time course is slightly different. In Figure 6 and in parallel determinations on material analyzed in Table 11, it was seen that the GA enhancement of organic phosphate radioactivity occurs only during hours 4 to 12. However the increase in the chemical level of organic phosphorus is not apparent at hour 6, but is present at hours 12 and 18. So the two effects overlap, but do not coincide exactly.

Data presented in this section, then, 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

In order 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 synthesis and is not merely a reflection of variations in the uptake and/or metabolism of ^{32}Pi.

In this work, 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.

In order 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 synthesis. Use of the ratio, phospholipid-^{32}P : 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 synthesis. 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 phospholipid synthesis can now be added to the series of events which preceed amylase production in barley aleurone layers and which can be directly related to protein synthesis. The response is initiated 4-6 hr after the addition of GA and increases to a maximum after 8-12 hr. This time course is in good agreement with the work of Evins (1971) 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 synthesis 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 recent work of Collins, et al. (1972) 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 synthesis 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 synthesis do not begin until after 4 hr of GA treatment.

The importance of phospholipid synthesis for the subsequent production of α-amylase and other hydrolases is demonstrated by the metabolic and hormonal control of ^{32}Pi incorporation into phospholipids. Cycloheximide rapidly inhibits any GA-enhanced increase in the rate of phospholipid synthesis without affecting the basal rate in control tissue. The same is true of 6-methylpurine which is known to have no effect on the rate of phospholipid synthesis in -GA layers (R.A.B. Keates, unpublished data) although it inhibits any GA-induced increases in this rate.

The role of abscisic acid as an antagonist of GA action has been documented for lettuce seed germination (Khan, 1968), germination of hazel seeds (Ross and Bradbeer, 1971), and in the aleurone layer system for inhibition of α-amylase production (Chrispeels and Varner, 1966), polysome formation (Evins and Varner, 1972), and production of lecithin-synthesizing enzymes (Johnson and Kende, 1971). Abscisic

acid also causes a rapid and specific inhibition of the GA enhancement of the rate of phospholipid synthesis. This inhibition is dependent on ABA concentration. Complete inhibition is obtained at 1 μM ABA which means that phospholipid synthesis is more sensitive to ABA than seed germination and is as sensitive as other processes in aleurone layers cited above.

Additional evidence for hormonal control of phospholipid synthesis is the requirement for the continued presence of GA. Removal of GA in mid-course results in a decrease in the rate of phospholipid synthesis. Moreover, this rate also depends on the concentration of GA in the incubation medium.

Furthermore, attempts at mimicking the GA stimulation of phospholipid synthesis failed. Cyclic AMP was not effective. Addition of amino acids to the incubation medium did not substitute for GA (unpublished results). Likewise, the addition of a series of compounds (glucose, amino acids, $MgCl_2$, $MnCl_2$, KCl, $CaCl_2$, glycerol, and inositol) which would be expected to be mobilized by early GA action did not stimulate phospholipid synthesis.

The inhibition of phospholipid synthesis by mannitol has several interesting aspects. The inhibition closely resembles the degree of inhibition of α-amylase production by mannitol and is specific for the GA-induced increase in the rate of phospholipid synthesis.

An additional effect of GA, the increase in the ratio, organic-^{32}P : ^{32}Pi uptake, is not affected by mannitol. This means that the relative increase in ^{32}Pi metabolism caused by GA is an early event not subject to regulation by osmotic stress. This is important because it is an example of a GA-induced process which can be physiologically separated from later GA-induced processes.

It has also been found (Koehler, et al., 1972) that mannitol does not inhibit to a significant extent the GA-induced increases in phosphorylcholine-cytidyl transferase (30% inhibition) or in phosphorylcholine-glyceride transferase (no inhibition). This clearly orders some of the events preceeding hydrolase induction. The increases in phospholipid synthesis and α-amylase production follow the increases in the activities of the lecithin-synthesizing enzymes. Between these events is a metabolic step which is sensitive to osmotic stress.

It has been proposed (Jones and Armstrong, 1971) that osmotic regulation is of physiological importance in controlling hydrolase production in germinating barley seeds. The effect of osmotic stress on α-amylase production may be via the inhibition of phospholipid synthesis. Alternatively, both phospholipid synthesis and hydrolase production may be inhibited because osmotic stress prevents the mobilization of precursors necessary for these processes (Koehler, et al., 1972; Jones, 1969 a).

There was no indication of a specific stimulation of phospholipid synthesis associated with the microsomal fraction of the aleurone cells. Phospholipid radioactivity had the same enhancement by GA in the 4,000 x g pellet, the 10,000 x g pellet and the 10,000 x g supernatant (Table 2). There is the possibility of some cross contamination of membranes in the cell fractions. Aleurone cells have very thick cell walls and the severe grinding procedures needed to insure all breakage could disrupt organelles. Nevertheless, the GA enhancement of phospholipid synthesis 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 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. 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 phosphorus or in acetate incorporation. The storage site of phospholipids could be the spherosomes which are present (Jones, 1969 c) in aleurone cells.

SUMMARY

Evidence for the hormonal control of phospholipid synthesis in the barley aleurone layer has been presented. The enhancement by GA of ^{32}Pi incorporation into phospholipids occurs throughout the cell. Inhibitors, whether metabolic, osmotic, or hormonal, which inhibit the synthesis of α-amylase have a similar effect on the rate of phospholipid synthesis. In all cases, the inhibition is specific for the GA enhancement of the basal rate of phospholipid synthesis. Thus the increase in the rate of phospholipid synthesis preceeds and is probably a necessary condition for the subsequent production of α-amylase and possibly other hydrolytic enzymes of the aleurone layer.

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