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Some Effects of Applied Gibberellic Acid on the Synthesis and Degradation of Lipids in Isolated Barley Aleurone Layers[1]

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ABSTRACT

An analysis of the lipids in isolated barley (*Hordeum vulgare* L.) aleurone layers after 12 hours incubation in the presence or absence of gibberellic acid showed no quantitative or qualitative changes. Longer incubation periods resulted in some lipid degradation which was greater in the presence of 1 μM gibberellic acid.

Glycerolipid synthesis was measured in isolated barley aleurone layers during the first 12 hours of incubation in the presence or absence of gibberellic acid by following the incorporation of ^{3}H-glycerol. No significant effect of the hormone was found on either the incorporation of glycerol into lipids or on the types of lipid being synthesized.

Barley aleurone layers respond to applied GA_3 by an increased production and secretion of hydrolases (21, 26, 27) which normally becomes evident 6 to 8 hr after hormone addition (3, 7, 10). The elucidation of events occurring during this 6- to 8-hr lag period has been the goal of a number of investigations (5, 7, 8), and attention has recently been focused on increased ER[3] synthesis (12). An enhanced incorporation of ^{14}C-choline into a semipurified membrane fraction was reported to occur 4 hr after GA_3 addition to aleurone layers (5), and the *in vitro* activities of PC-cytidyl transferase and PC-glyceride transferase were also found to be increased during the lag period (11). GA_3-enhanced incorporation of ^{32}P into phospholipids, however, only became evident 4 to 5 hr after the start of GA_3 treatment, and it was suggested that the increased ^{32}P incorporation might have reflected an increased turnover rather than an increased net synthesis of phospholipids (15, 16).

Before investigating in greater detail the effect of GA_3 on lipid synthesis or turnover during the lag period, it was felt desirable to establish which lipids were influenced by hormone treatment, and to determine more precisely how the over-all pattern of lipid synthesis changed.

MATERIALS AND METHODS

Preparation of Barley Aleurone Layers. Barley (*Hordeum vulgare* L. cv. Himalaya, 1967) aleurone layers were prepared as described by Chrispeels and Varner (3) and incubated under the same conditions as by Johnson and Kende (11). For studies on the incorporation of ^{3}H-glycerol into lipids, Himalaya barley of the 1969 harvest was used; when layers of these seeds were prepared and incubated as above, they gave a poor GA_3 response because of high α-amylase production in the absence of the hormone. The substitution of 20 mM sodium succinate buffer of pH 5.3 (4) for the more commonly used 2 mM sodium acetate buffer of pH 4.8 restored the hormonal response by reducing α-amylase production in the absence of GA_3 and increasing the enzyme production in the presence of the hormone (1 μM). The preincubation conditions were also changed to advantage by imbibing 50 half-seeds in 9 ml of 20 mM succinate buffer (pH 5.3) containing 20 mM $CaCl_2$ on 9-cm Whatman No. 3 filter paper, instead of on moist sand. This resulted in less endosperm liquifaction during the imbibition period of 3 to 4 days.

Extraction of Lipids. Twenty-five to fifty layers were ground to a powder in liquid nitrogen and then extracted with 25 ml of chloroform-methanol (2:1, v/v) for 30 min at 4 C. The extract was filtered and the residue reground and again extracted with 15 ml of chloroform-methanol (2:1, v/v) for 30 min more. After filtration, the extracts were combined and shaken with 0.2 vol 0.73% (w/v) NaCl (6). The lower, lipid-containing phase was collected, the solvent removed by distillation under reduced pressure, and the residue (lipids) weighed.

Fractionation of the Lipids. Preliminary experiments using two-dimensional TLC to analyze the individual lipids were unsuccessful due to the large amount of neutral lipid in the extract, the presence of which limited the amount of phospholipids we could apply to a plate without overloading it. A prepurification of the total lipid extract was thus undertaken using DEAE-cellulose (Whatman DE-52) column chromatography as described by Roughan and Batt (22), the extract being separated into three fractions. The neutral fraction, eluted with chloroform, contained less than 1.5% of the total lipid phosphorus and consisted mainly of triglycerides, diglycerides, monoglycerides, sterols, sterol esters, and hydrocarbons. The nonacidic fraction, eluted with chloroform-methanol (6:4, v/v), usually contained 70 to 75% of the total lipid phosphorus and its major components were PC, PE, and LPC. The acidic fraction, eluted with chloroform-methanol (6:4, v/v) containing 0.2% (w/v) ammonium acetate and 2% (v/v) 14 N NH_4OH, contained 25 to 30% of the total lipid phosphorus, and the major compounds present were PI and PG with four other, unidentified substances.

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[3] Abbreviations: ER: endoplasmic reticulum; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PG: phosphatidylglycerol; LPC: lysophosphatidylcholine; DPG: diphosphatidylglycerol.

TABLE I. *Level of Extractable Lipids in Barley Aleurone Layers after Incubation for Varying Times with and without GA_3*

Total and neutral lipids measured by weighing and lipid phosphorus by colorimetric analysis (23). Each value is the average of two replicates.

	Exp. I			Exp. II		
	0-hr control	12-hr control	12-hr GA_3	0-hr control	24-hr control	24-hr GA_3
Total lipid extract (mg 50 layers)	22.1	20.0	19.3	23.8	17.8	13.9
Total neutral lipids (mg 50 layers)	18.1	16.2	17.8		10.2	7.6
Total lipid phosphorus (μmole 50 layers)	4.1	3.8	3.8	4.0	3.4	2.6

TLC of the Lipid Fractions. Glass plates (20 × 20 cm) were coated with 0.25 to 0.30 mm layers of silica gel (Merck G or GF_{254}) and after drying were activated for 30 min at 110 C. The nonacidic lipids were separated with chloroform-methanol-14 N NH_4OH (65:30:4, v/v) and the acidic lipids with chloroform-methanol-acetic acid-H_2O (85:15:10:3, v/v) as developing solvents. Compounds were identified by co-chromatography with known lipid standards and by characteristic reactions to spray reagents (25).

Phosphorus Analysis. Aliquots of an extract or zones from a TLC plate were analyzed by the method of Rouser *et al.* (23).

Fatty Acid Analysis. An aliquot of the lipid extract was added to 4 ml of methanol-benzene-H_2SO_4 (15:7.5:1, v/v) and heated for 3 hr at 80 C. Water (10 ml) was added to the mixture, and the fatty acid methyl esters were extracted from the aqueous layer with petroleum ether (b.p. 40–60 C). The fatty acids were analyzed by GLC using a 6-ft 10% DEGS column at 170 C.

Studies with ^{3}H-Glycerol. 2-^{3}H-Glycerol with a specific radioactivity of 500 mCi/mmole was purchased from Amersham/Searle and was stored in ethanol at −20 C. 1-^{14}C-Glycerol was purchased from the same supplier and had a specific radioactivity of 26 mCi/mmole.

2-^{3}H-Glycerol was added directly to the incubation flask (normally 1–5 μCi/25 layers), with or without carrier glycerol, and its incorporation into lipids was measured after 45 min. The rate of incorporation was constant up to 90 min, the longest incubation period used. After the labeling period, the layers were washed with distilled water, with 10 mM glycerol and blotted dry before being stored at −20 C or being extracted immediately as described above. The total lipid extract after Folch extraction was washed at least once with Folch upper phase containing 1 mM glycerol.

The distribution of ^{3}H between polar glycerolipids, 1,2-diglycerides and triglycerides was determined by analyzing an aliquot of the total lipid extract by TLC on silica gel G with petroleum ether-ether-acetic acid (70:30:2, v/v) as developing solvent. More than 95% of the ^{3}H was found in the combined zones corresponding to triglycerides, 1,2-diglycerides, and polar glycerolipids. The latter remained at the origin of the chromatogram.

Radioactivity in the total lipid extract was determined by liquid scintillation counting, an aliquot of the sample in toluene/ethanol (1:1, v/v) being added to a toluene-based scintillation mixture (toluene 3000 ml, PPO 12 g, POPOP 300 mg). Radioactivity on a TLC plate was assessed by scraping the relevant silica-gel areas into scintillation vials, adding 0.5 ml H_2O and 10 ml of a dioxan-based scintillation mixture (naphthalene, 100 g; PPO, 5 g; dimethyl POPOP, 0.3 g; dioxan, 730 ml; toluene, 135 ml; and 35 ml of methanol). Using this procedure, recovery of ^{3}H from ^{3}H-labeled total lipid extract applied to silica gel was greater than 95%.

Glycerol Determinations. Ten layers (Himalaya, 1969) were incubated in succinate buffer for the appropriate times and, after removal from the incubation media, were washed with distilled water, blotted, and ground in 2 ml of iso-propyl alcohol in a mortar. The extract was poured into a 12-ml centrifuge tube, 3 ml of ethanol being used to rinse the mortar and pestle. The extract was centrifuged, the alcoholic supernatant solution removed, and the residue subsequently re-extracted with 3 ml of ethanol. The alcoholic extracts were combined, dried under reduced pressure, and taken up in 1 ml of distilled water. The extracts were applied to small columns (8 × 0.5 cm) of Dowex 1-X8 (formate) ion exchanger; the columns were eluted with 6 ml of distilled H_2O, the effluent was concentrated, and glycerol was determined by the method of Chernick (1).

RESULTS

Lipids and Phospholipids in Aleurone Layers Incubated with and without GA_3. An analysis of the amount of total lipid, neutral lipid, and lipid phosphorus in extracts of isolated barley aleurone layers, incubated for 12 or 24 hr in the presence or absence of 1 μM GA_3, showed no net synthesis of any class of lipid at 12 hr. After 24 hr, the level of lipids decreased in aleurone layers without GA and even more in those incubated with the hormone (Table I).

A more detailed examination of the major phospholipids present in the barley aleurone before and after incubation in GA failed to demonstrate any significant hormonal effect on the level of individual phospholipids (Table II). Thus, if ER synthesis was occurring during the lag period (11, 12), the endoplasmic reticulum synthesized must have had a phospholipid composition very similar to the average composition of the cell or it must have represented a very small fraction of the cell phospholipids.

The spectrum of fatty acids in different cell membranes differs (20), and cells in various stages of differentiation can show altered fatty acid compositions (17). A change in the relative proportions of the different cell membranes might be reflected in an altered spectrum of the fatty acids in barley aleurone cells. An analysis of the fatty acid composition of the total lipid extract after 24 hr incubation in the presence or absence of the hormone, however, showed no significant changes and the fatty acid compositions of PC and PE from the same samples were also unchanged in the presence of GA_3 (data not shown).

Table II. *Relative Levels of Major Phospholipids in Barley Aleurone Layers after Incubation for 12 hr in Absence or Presence of GA_3*

Each value is the average of two replicates.

Phospholipid	Total Lipid Phosphorus		
	0-hr control	12-hr control	12-hr GA_3
	%		
PC	45.7	46.6	45.3
PE	11.6	11.0	10.6
PI	13.0	13.4	12.7
LPC	4.3	3.3	3.1
PG (?)	2.7	3.8	4.5
Others (4)	<5% each	<5% each	<5% each

Table III. *Effect of GA_3 on Incorporation of ^3H-Glycerol into Total Lipid Fraction in Presence of Unlabeled Glycerol*

The layers were incubated for 4 hr with or without GA_3 and then for 45 min in the presence of ^3H-glycerol with or without GA_3, respectively. Results from replicated treatments shown.

Glycerol Concn in Incubation Medium	Glycerol Incorporated	
	$-GA_3$	$+GA_3$
mM	*nmoles/hr·10 layers*	
1	15.1, 14.3	19.4, 14.4
10	42.9, 47.3	51.9, 45.8

Incorporation of ^3H-Glycerol into Lipids in Aleurone Layers Treated with and without GA_3. It was thus apparent that incubation of barley aleurone layers in buffer alone resulted in a slight decline in over-all lipid levels; this decline was accelerated by GA_3. None of the individual phospholipids analyzed was selectively retained or degraded, however. At this stage of the investigation, the possibility that GA stimulated lipid synthesis during the lag period was re-evaluated, studying ^3H-glycerol incorporation into glycerolipids. It had previously been shown that ^3H-glycerol was an adequate lipid precursor for membrane synthesis (2). Layers incubated in the presence or absence of GA_3 for periods up to 12 hr were tested for their ability to incorporate ^3H-glycerol into lipids. In an attempt to ensure that glycerol incorporation was being studied at saturating substrate concentrations, carrier glycerol was added to the ^3H-glycerol in increasing amounts to give final glycerol concentrations in the incubation medium of 0.1 μM to 10 mM. Glycerol added up to 1 mM did not reduce the incorporation of ^3H-glycerol, but at 10 mM a marked reduction in incorporation of the precursor was noted, suggesting that the endogenous glycerol content of the layers was in the order of 1 to 10 mM.

Thus, any GA_3 effect on the glycerol pool size was obviated by investigating the effect of the hormone in the presence of 10 mM glycerol. Such an experiment was conducted (Table III), and it was evident that the hormone did not significantly increase the capacity of barley aleurone layers to synthesize glycerolipids from glycerol during the lag phase.

Freshly isolated aleurone layers from 1969 Himalaya barley contain approximately 1.3 μmole glycerol/50 layers (1 layer = about 15 mg fresh weight). During the first 4 hr of incubation, the level of glycerol declined by approximately 50%, a decline not significantly influenced by treatment with GA_3. Estimating that freshly isolated layers are approximately 75% water and that glycerol is evenly distributed, the endogenous glycerol concentration would be around 2.5 mM. The rate of glycerolipid synthesis in layers 4 hr after isolation was 15 to 20 nmole/10 layers·hr in the presence of 1 mM glycerol, and at saturating substrate concentrations (10 mM glycerol) the rate was 40 to 50 nmole/10 layers·hr (Table III); hence, it seems unlikely that glycerolipid synthesis from endogenous glycerol would exceed 30 nmole/10 layers·hr during the lag period. Since phospholipids account for approximately one-third of the incorporated glycerol (Tables IV, V, and VI), it can therefore be estimated that phospholipid synthesis did not exceed 10 nmole/10 layers·hr. An analysis of the distribution of radioactivity from ^3H-glycerol in the major lipid classes was undertaken, and it was consistently found that the presence of GA_3 increased the incorporation of ^3H-glycerol into nonpolar lipids relative to polar lipids (Table IV). An analysis of the ^3H-glycerol incorporation into the phospholipid fraction showed that GA_3 did not have any marked effect on the types of phospholipids being synthesized (Tables V and VI).

The possibility that the dihydroxyacetone pathway (9) was contributing to over-all glycerolipid synthesis, thus masking a hormone effect on the glycerol-3-phosphate pathway, was briefly investigated in a double-label experiment using 2-^3H-glycerol and 1-^{14}C-glycerol. If lipid synthesis was occurring via the dihydroxyacetone pathway, one might expect a decrease in the ratio of $^3H/^{14}C$ in the total lipid extract compared to the $^3H/^{14}C$ ratio of the glycerol added to the layers, because of a

Table IV. *Effect of GA_3 on Incorporation of ^3H-Glycerol into Polar Glycerolipids, Diglycerides, and Triglycerides*

Barley aleurone layers were incubated for 10 hr with or without GA_3, and then labeled with ^3H-glycerol for 45 min. The total lipid extracts were analyzed by TLC, each replicated treatment being analyzed twice. The results are the average of four analyses, at least 3000 dpm being analyzed on each TLC plate.

	Incorporated ^3H-Glycerol in Each Class of Lipid	
	$-GA_3$	$+GA_3$
	%	
Triglycerides	26	33
Diglycerides	33	38
Polar glycerolipids	41	29

Table V. *Effect of GA_3 on Synthesis on Nonacidic Phospholipids in Barley Aleurone Layers*

Layers were incubated for 12.75 hr with or without GA_3, the last 45 min in the presence of ^3H-glycerol. Nonacidic lipids were recovered from the total lipid extracts after DEAE-column chromatography and were further analyzed by TLC. Approximately 5 to 10% of the ^3H-glycerol incorporated into the total lipid extract was incorporated into nonacidic phospholipids and aliquots containing in excess of 5000 dpm were analyzed by TLC.

	^3H in the Analyzed Fraction as a % of ^3H in the Total Nonacidic Fraction	
	$-GA_3$	$+GA_3$
PC	73	77
PE	7.1	6.5
LPC	2.1	1.4
Unidentified 1	7.6	7.2
Unidentified 2	1.4	3.6

Table VI. *Effect of GA_3 on Synthesis of Acidic Phospholipids in Barley Aleurone Layers*

Procedure as given in Table V. Approximately 20 to 30% of the ^3H-glycerol incorporated into the total lipid fraction was found in the acidic-phospholipid fraction and aliquots analyzed by TLC contained in excess of 20,000 dpm.

	^3H in Analyzed Fraction as a % of ^3H in the Total Acidic-phospholipid Fraction	
	$-GA_3$	$+GA_3$
PI	25	25
Unknown 3	3	3
Unknown 4	66	64
Unknown 5	5	7

loss of ^{3}H from 2-^{3}H-glycerol on conversion to dihydroxyacetone (18). In fact, the ratio $^{3}H/^{14}C$ in the total lipid extract of layers labeled with a mixture of 2-^{3}H glycerol and 1-^{14}C-glycerol increased slightly and was not influenced by the presence of GA_3.

DISCUSSION

The evidence obtained in the present investigation would suggest that lipid synthesis in barley aleurone layers is not radically changed by the presence of GA_3 during the lag period. No increase in lipid content was found in layers analyzed 12 hr after the addition of the hormone, and measurement of the incorporation of glycerol into glycerolipids showed no increased capacity of this pathway during the lag period. The rate of phospholipid synthesis from glycerol is not such that any significant change in phospholipid composition could be expected during the lag period, an estimated 10 nmole phospholipid/hr·10 layers being synthesized in aleurone cells which contain 700 to 800 nmoles phospholipid/10 layers initially.

Three previous investigations have suggested that GA_3 stimulates phospholipid synthesis in barley aleurone layers (5, 11, 15, 16). The result of the present investigation, and the finding that GA_3 does not increase ^{14}C-acetate incorporation into the lipid fraction of barley aleurone layers (16) suggests that the hormone does not increase lipid synthesis. The conflicting results could be partially explained if 1,2-diglycerides, existing or produced from triglycerides, serve as precursors for the synthesis of phospholipids, the production of 1,2-diglycerides from glycerol and fatty acids not being required. If this were true, one could expect substantial ^{32}P incorporation into phosphatidic acid, the probable precursor of PI and PG, as a result of diglyceride kinase activity (19); this was not borne out in experiments of Koehler and Varner (16) who found little ^{32}P incorporated into phosphatidic acid. The lack of ^{32}P incorporation into phosphatidic acid in barley aleurone layers contrasts with the situation in immature soybean where phosphatidic acid is the most highly labeled compound after feeding $Na_2H^{32}PO_4$ for short periods of time (24).

If phospholipid synthesis was occurring in barley aleurone cells utilizing neutral lipid precursors, one would expect the rates of lipid synthesis measured by ^{32}P incorporation or by ^{14}C-choline incorporation to exceed the rates of lipid synthesis measured by ^{3}H-glycerol incorporation. Unfortunately, in neither of the *in vivo* studies where a GA_3 stimulation of lipid synthesis was reported (5, 16) were the actual rates of lipid synthesis measured. Instead, the incorporation of labeled precursor from an unmeasured pool was recorded. If an estimate of the pool sizes encountered in the work of Evins and Varner (5) and Koehler and Varner (16) is made, however, it would seem that the rates of lipid synthesis in both studies were not greatly different from those measured in the present study. The *in vitro* enzyme activities measured by Johnson and Kende (11) are not helpful in assessing the rate of phosphatidyl choline synthesis *in vivo* because of the greatly differing rates of recovery of the three enzymes studied. The *in vitro* activity of choline kinase, which was not influenced by GA_3 treatment of the layers, was 2 to 4 nmole/hr·10 layers, an activity consistent with the results of the present study. The *in vitro* activities of the two membrane-bound enzymes, which were affected by hormone treatment of the layers, were low, however, and would account for only 1 to 10% of the rate of *in vivo* synthesis of PC measured in this study. The GA_3 effect on the *in vitro* activity of these enzymes began at least 2 hr earlier than the reported hormonal effects on ^{14}C-choline or ^{32}P-orthophosphate incorporation into lipids, and the possibility must be considered that the *in vitro* assay of these enzymes, whilst detecting an interesting GA effect, does not reflect *in vivo* enzyme activity.

Finally, the evidence that GA_3 stimulates ER accumulation by a direct effect on membrane synthesis must be reconsidered. No unequivocal biochemical evidence has yet been offered that GA_3 specifically stimulates the synthesis of any membrane fraction in barley aleurone cells, and electron-microscopic evidence has not yet yielded quantitative data despite the publication of a promising method (14). Applied GA_3 causes changes in other membranes apart from the ER (12, 13) and it is possible that these changes are more relevant to the GA_3 effect on ^{32}P incorporation. The apparent ER accumulation might result from the utilization of intact membrane constituents stored elsewhere in the cell and transferred to the developing ER. Such a transfer of intact lipids between existing membranes has been demonstrated in plants (19) although its significance is uncertain.

In conclusion, in the present and previous investigations no GA_3 effect on lipid synthesis has been shown in barley aleurone layers during the first half of the lag period and the relevance of any subsequent changes to the release of hydrolases is uncertain.

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