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Gibberellic Acid and Ion Release from Barley Aleurone Tissue

EVIDENCE FOR HORMONE-DEPENDENT ION TRANSPORT CAPACITY

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ABSTRACT

The release of potassium, magnesium, and phosphate ions from aleurone cells of barley (*Hordeum vulgare* L. cv. Himalaya) is a gibberellic acid-dependent process. The release of these ions is preceded by a lag period of 6 to 8 hours after gibberellic acid addition. The effect of gibberellic acid on the release of ions is not mediated through an effect on ion solubilization. Thus, gibberellic acid does not apreciably affect the sum of extracted and released ions relative to controls. Rather, the effect of the hormone is on the release process itself. Inhibitors of oxidative phosphorylation when added with gibberellic acid or at times up to 6 hours after gibberellic acid inhibition release. When these inhibitors are added after ion release has begun, however, rapid efflux of ions occurs. These results suggest a strong correlation between energy levels and ion transport capacity. Inhibitors of RNA and protein synthesis also inhibit gibberellic acid-stimulated ion release. Evidence suggests that RNA and protein synthesis are required to establish and maintain ion release capacity of aleurone cells.

The GA enhancement of hydrolytic enzyme synthesis at the expense of reserve proteins in aleurone layers of barley is firmly established (17, 24, 27). Of those enzymes whose activities are enhanced by GA, at least four are synthesized *de novo* by the aleurone cell (1, 8, 11). In addition to protein, aleurone cells store other reserves, notably lipid and phytin. However, in contrast to the information available on the role of proteins in barley aleurone cells, little is known about phytin and lipid metabolism.

Phytin (Ca, Mg salt of inositol hexaphosphate) is presumed to be the principle reserve of phosphorous, magnesium, calcium, and possibly potassium in cereal seeds (16, 21, 25). In barley, phytin contains about one-half of the total phosphorous present in the seed (7). Whether calcium and magnesium ions are always associated with phytin is in question (22). Sandegren (22) reported that in mature barley grain, phytin exists as the water-soluble sodium-potassium salt which, after steeping, undergoes conversion to the insoluble calcium-magnesium salt by ion-exchange.

In wheat most of the phytin of the grain is located in the aleurone layer (15). Similarly, as much as 60% of the inorganic ions of the seed are found in this tissue (9). A definitive analysis of the distribution of phytin and inorganic ions has not been made in barley. Phytin does, however, occur in aleurone cells of barley as shown by the presence within protein bodies of distinct electron dense globoids (4, 10, 12, 18).

During malting of barley, an 8-fold increase is found in the level of phytase, the enzyme responsible for the hydrolysis of phytin to myoinositol and inorganic phosphate (7). De-embryonated half seeds of barley also possess phytase activity which, as in wheat, is mostly in the aleurone layer (19, 20, 23). GA treatment increases phytase activity in half seeds of barley; however, the effect of GA is low when compared with its effects on other hydrolases (2, 23). Since a large proportion of the phosphate, magnesium, and calcium of the barley seed may be present in phytin, the redistribution of these elements would be dependent on the hydrolysis of phytin by phytase and the subsequent release of these elements from the aleurone cell (25, 26).

In experiments reported in this paper, emphasis has been placed on the role of GA in the release of minerals from the isolated aleurone layer of barley.

MATERIALS AND METHODS

Plant Material and Experimental Design. Barley (*Hordeum vulgare* L. cv. Himalaya, 1969 harvest) seeds were prepared according to the methods previously described (2, 13). Aleurone layers were stripped from de-embryonated half seeds which had imbibed on moist, sterile sand for 3 days. Aleurone layers were incubated in water containing 20 mM $CaCl_2$ and the appropriate GA concentration. Two basic experimental designs were used, depending on the parameter to be measured. When determining the time course of ion release, 50 aleurone layers were incubated in 150-ml Erlenmeyer flasks containing 25 ml of incubating solution. Aliquots of the medium (50 μl) were removed at hourly or half-hourly intervals. Amylase and phosphate levels were determined in each aliquot without further dilution. Cations were measured after appropriate dilution with deionized distilled water. For determination of extractable ions, five aleurone layers were incubated in 25-ml Erlenmeyer flasks containing 2 ml of medium. At each sampling time, three flasks were analyzed for every treatment.

Determination of α-Amylase and Ions. α-Amylase was determined using the starch-iodine procedure described previously (13). Magnesium, potassium, and phosphate were determined in both tissue extracts and incubation medium. Aleurone layers were ground in a glass homogeniser with cold (2 C) deionized distilled water. The homogenate was centrifuged at 2000*g* to remove particulate and insoluble materials. The supernatant contains those ions which are not chelated by phytic acid and are referred to in the text as free, soluble ions. It should be noted that this description refers only to the fact that these ions are soluble in cold water and are thus not complexed with a highly insoluble organic compound. Magnesium and potassium were determined by atomic absorption spectrophotometry. Phosphate was assayed using the ammonium molybdate-semidine (N-phenylenediamine hydrochloride) procedure. The blue complex produced was measured at 625 nm.

Continuous monitoring of potassium in the incubation was accomplished with a glass potassium-responsive electrode (Electrical Industries Limited, England; type BH 115). The

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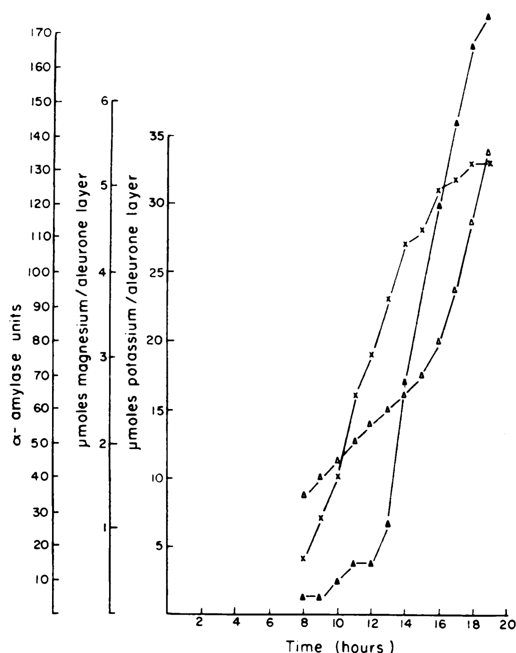

FIG. 1. Time course of potassium (×), magnesium (△), and α-amylase (▲) release from 50 aleurone layers incubated in 25 ml of 5 μM GA and 20 mM $CaCl_2$.

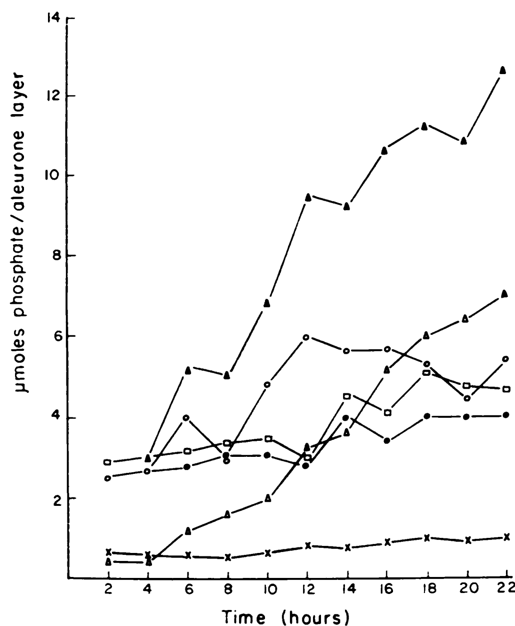

FIG. 2. Effect of 5 μM GA on released (△, ×), extracted (○, ●), and total (▲, □) phosphate. △, ○, ▲: GA; ×, ●, □: water controls.

electrode was connected to a standard Beckman pH meter whose signal output was fed directly to a strip chart recorder.

RESULTS

The release of magnesium, potassium, phosphate, and α-amylase from isolated aleurone layers into a solution of 20 mM $CaCl_2$ was followed with time. In the absence of GA, aleurone layers released little potassium, magnesium, or phosphate into the surrounding incubation medium (Figs. 2 and 3). Addition of GA to the incubation medium, however, causes the release of these ions to the surrounding medium (Figs. 1 and 2). The time course of release of potassium, magnesium, and α-amylase differ significantly (Fig. 1). Of the total amount of potassium released over a 19-hr period after GA treatment, 50% was released by 11 hr, while for magnesium 50% of the total ion was released only after 16 hr (Fig. 1). The release of potassium and magnesium from aleurone layers, like α-amylase, is dependent on the concentration of GA (Table I).

Omission of calcium from the incubation medium has no effect on the release of cations, although it is required to prevent the irreversible inactivation of released α-amylase (Table II).

Imbibed aleurone layers contain appreciable levels of soluble phosphate, potassium, and magnesium (Figs. 2 and 3). GA has little effect on the sum of the extractable and released potassium in aleurone layers (Fig. 3). The primary effect of GA on potassium is therefore on its release into the surrounding medium (Figs. 1 and 3). There is an increase, however, in the sums of soluble and released magnesium and phosphate after GA application (Figs. 2 and 3). Thus both ion release and ion solubilization are GA dependent processes.

We have determined some of the physiological parameters governing these processes. MCCP[1], an inhibitor of oxidative phosphorylation which also affects membrane systems, inhibits cation release when it is given with or 6 hr after GA (Fig. 4). However, when this inhibitor is given 12 hr after GA, it causes a rapid release of cations (Fig. 4). Sodium azide, cyanide, and 2,4-dinitrophenol also inhibited cation release when given with GA and also increased ion release when given at 10 to 12 hr after GA. MCCP and sodium azide also inhibit phosphate release when given with GA and increase its release when given 10 hr after GA (Table III). The effect of MCCP on the sum of extractable and released potassium and magnesium is shown in Figure 3. The rapid efflux of these cations is associated with an equal decrease in the level of extractable cations (Fig. 3). MCCP does not affect the sum of the extracted and released potassium in these cells, although a small increase in the sum of the extracted and released magnesium is seen (Fig. 3).

The time course of potassium release from aleurone cells after sodium azide and MCCP treatment was determined. Aleurone layers were incubated in a medium containing 20 mM $CaCl_2$ and 5 μM GA for 10 to 12 hr. After a constant rate of potassium efflux was attained, MCCP or azide was added to the incubation flask, and the potassium efflux was monitored for a further period of time. Rapid potassium efflux was initiated within 5 to 6 min of inhibitor addition and persisted at an elevated rate for at least 25 min (Figs. 5 and 6).

The GA-controlled efflux of ions from aleurone cells is a temperature-dependent process (Table IV). Thus, with increased temperature, there is an increase in the amount of the ion in the medium (Table IV). In contrast to α-amylase, ion release is not inhibited at temperatures above 30 C (Table IV).

The effects of inhibitors of protein and RNA synthesis on the development of the ability of these cells to release ions was also tested. Cycloheximide is effective in inhibiting α-amylase production when given with GA or at times up to 12 hr after GA (Fig. 7). Cycloheximide is also an effective inhibitor of potassium and magnesium efflux when given with GA or within 5 hr after GA (Fig. 7). If cycloheximide addition is delayed for 9 hr after GA, it is less effective in inhibiting potassium efflux, and if its addition is delayed 12 hr, it is without effect (Fig. 7).

[1] Abbreviation: MCCP: carbonyl cyanide *m*-chlorophenol hydrazone.

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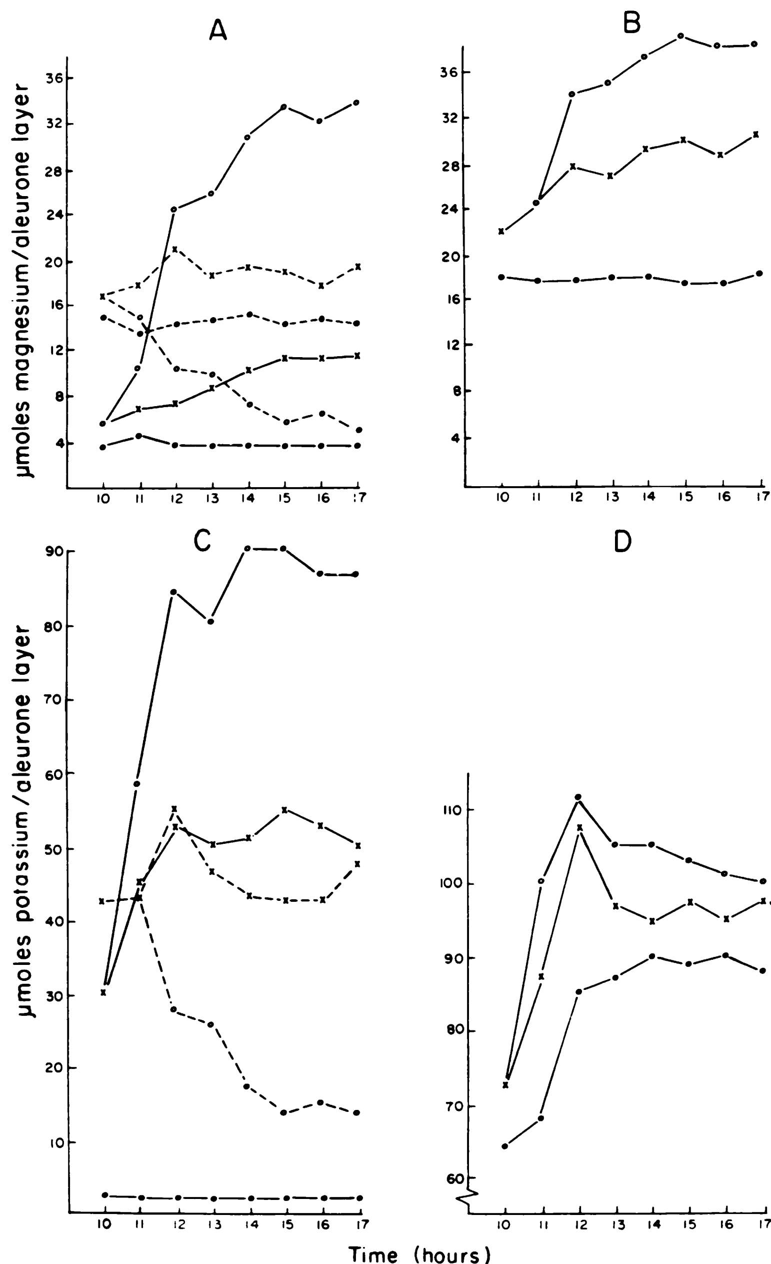

FIG. 3. Relationship between released, extractable, and sum of soluble and released magnesium (A, B) and potassium (C, D). ×: H_2O; ○: 5 μM GA; ●: 5 μM MCCP; - - -: extract; ——: medium and total. A and C: medium and extract; B and D: total level of ions.

For magnesium release, addition of cycloheximide as much as 12 hr after GA addition is still effective in reducing its release into the outside medium (Fig. 7).

6-Methylpurine, an inhibitor of RNA synthesis, is also an effective inhibitor of cation efflux, if it is added with GA. If 6-methylpurine is delayed for 5 hr after GA addition, the efflux of potassium is only slightly affected, whereas a delay of 9 hr or greater in 6-methylpurine addition is without effect on potassium release (Fig. 8). Magnesium efflux and α-amylase production are inhibited by 6-methylpurine in similar ways (Fig. 8).

DISCUSSION

The storage of phosphate and cations in a complex with myoinositol in cereal seeds is now widely accepted (4, 7, 12, 15, 16, 18, 25, 26). In wheat, the mobilization of these elements is known to occur during germination when the embryo accumulates these ions at the expense of the aleurone layer (3). During the mobilization of these ions at least two processes can be distinguished. First, the ion-inositol complex must be hydrolyzed by phytase, and second, the cations and inorganic phosphate which are liberated must be released to the milieu of the endosperm for transport to the seedling via the scutellum.

We have shown that the rate of release of these cations and phosphate from the aleurone layer is increased by GA with a lag time of 6 to 8 hr (Figs. 1, 2, and 3). Two possible interpretations of this release are that ion efflux is specifically controlled by GA, and that ion efflux is a passive, nonspecific loss of these ions. Epstein (5, 6, 14) has emphasized that experiments on ion movement should be interpreted with caution, particularly those experiments where calcium is omitted from

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Table I. *Effect of GA Concentration on α-Amylase, Potassium, and Magnesium Release from Aleurone Layers*

Treatment[1]	Potassium	Magnesium	
	μmoles/aleurone layer		*units α-amylase*
H_2O	3.0	1.2	21.0
GA 5×10^{-10}	3.5	2.3	31.0
GA 5×10^{-8}	35.0	5.7	178.0
GA 5×10^{-6}	48.5	9.0	372.0

[1] Fifty aleurone layers incubated for 20 hr at 25 C in 25 ml of buffer containing the appropriate GA concentration.

Table II. *Effect of Calcium on Ion and α-Amylase Release*

Treatment[1]	Potassium	Magnesium	
	μmoles/aleurone layer		*units α-amylase*
$H_2O + CaCl_2$	2.5	2.0	36.0
$H_2O - CaCl_2$	2.5	1.9	15.0
GA + $CaCl_2$	34.5	12.0	660.0
GA − $CaCl_2$	30.0	11.7	96.0

[1] Fifty aleurone layers incubated for 20 hr at 25 C in 25 ml of buffer containing 5 μM GA, and where appropriate, 20 mM $CaCl_2$.

Table III. *Effect of MCCP and Azide on Phosphate Release*

Treatment[1]	
	μmoles p/aleurone
H_2O	2.9
GA 5 μM	6.1
GA + 5 μM MCCP	10.3
GA + 5 mM NaN_3	10.0

[1] Experimental conditions as in Table I.

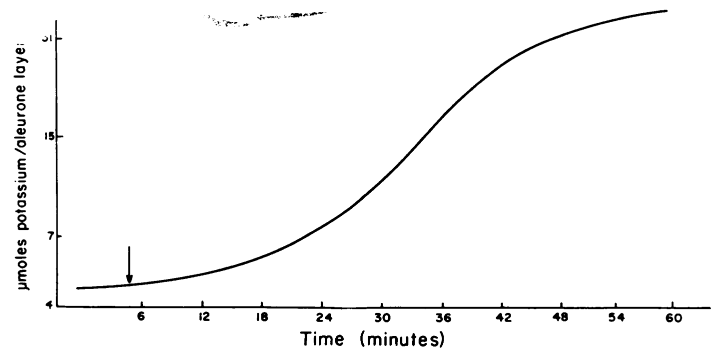

FIG. 5. Effect of MCCP on the rate of potassium efflux from aleurone cells. Fifty aleurone layers were incubated in 25 ml of 5 μM GA for 10 hr. MCCP (5 μM) was added to aleurone layers at 10 hr (arrow), and the rate of efflux was monitored with a potassium-responsive electrode.

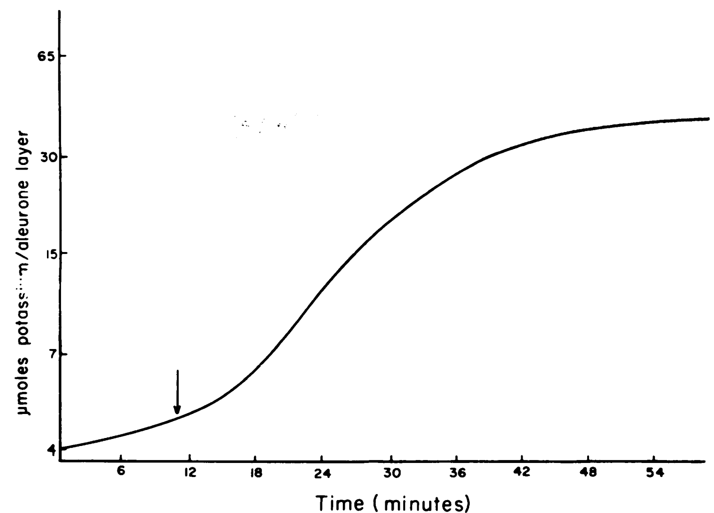

FIG. 6. As Fig. 5, except 5 mM sodium azide was added 10 hr after GA (arrow).

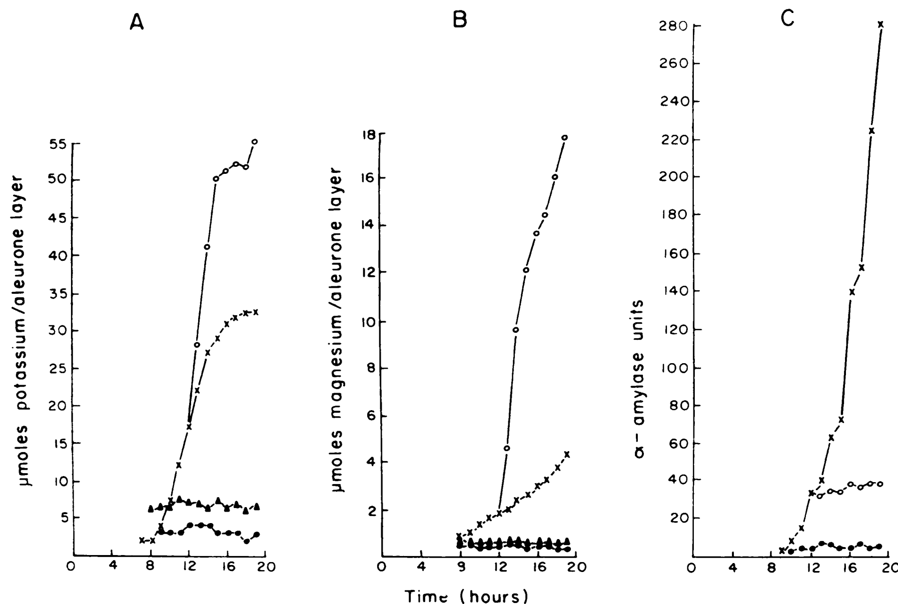

FIG. 4. Effect of MCCP on potassium (A), magnesium (B), and α-amylase (C) release from aleurone cells. Fifty aleurone layers were incubated in 20 mM $CaCl_2$ with 5 μM GA and inhibitor addition. ●: H_2O and inhibitor given with GA at time 0; ×: GA; ▲: inhibitor given 6 hr after GA; ○: inhibitor given 12 hr after GA.

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the ambient solution. Lauchli and Epstein (14) stress that "calcium is essential for the integrity of root cells and especially the plasmalemma." Thus, it could be imagined that GA increases ion efflux from aleurone cells by reducing the endogenous calcium level thus affecting membrane permeability. We find that GA stimulates ion release both in the presence and absence of exogenous calcium (Table II).

The role of GA in causing ion release could be indirect; thus, GA could increase phytase activity making available more ions for release. This role of GA in causing cation efflux is not supported by the evidence. GA does not affect the level of total potassium relative to the control and has only a small effect on the level of total magnesium. (Fig. 3). If GA affected release of ions by making more of these ions available for release, then the sums of the extractable and released ions would increase relative to the water controls (Fig. 3).

The sum of the extractable and released phosphate is increased by increasing GA concentrations (Fig. 2). This increase could be related to an inhibition of the activity of phytase or other phosphatases by phosphate. The inhibition of phosphatase activity by inorganic phosphate is of general occurrence and has been widely reported in seeds (7). Release of phosphate ions from the aleurone layer would relieve the inhibition of hydrolysis thus allowing for an increase in extractable phosphate (Fig. 2). That GA does not affect ion release by increasing phytase activity is also suggested by the work of Eastwood and Laidman (3) in wheat. These workers showed that when the embryo was replaced with GA an increase in ion efflux from wheat aleurone cells occurred without a concomitant increase in phytase activity (3).

Table IV. *Effect of Temperature on Release of Potassium, α-Amylase, and Magnesium*

Treatment[1]	Potassium	Magnesium	
	μmoles/aleurone layer		units α-amylase
2 C			
H_2O	3.5	1.9	12.0
GA	30.0	4.9	123.0
12 C			
H_2O	3.0	2.1	12.0
GA	37.5	6.6	130.0
25 C			
H_2O	3.4	2.1	13.0
GA	43.0	9.0	460.0
35 C			
H_2O	2.5	3.3	4.0
GA	47.5	11.9	185.0

[1] Experimental conditions as in Table I. GA at 5 μM.

Experiments with inhibitors of oxidative phosphorylation also indicate that ion availability is not the regulator of ion release. Thus, when aleurone layers are treated with MCCP, azide, 2,4-dinitrophenol, or cyanide after cation release has begun, a 10-fold increase in rate of ion release is observed (Figs. 4, 5, and 6). This marked increase in the rate of ion release is not paralleled by any increase in the rate of production of free cation (Fig. 3). On the contrary, with an increased rate of ion efflux there is a sharp decline in the level of extractable cations. If the availability of free ions regulated ion efflux, then the phase of rapid ion efflux induced by inhibitors of oxidative phosphorylation would be associated with a marked increase in the sum of extractable and released cation (Fig. 3).

The mechanism whereby inhibitors of oxidative phosphorylation increase the efflux of ions is not clear. If GA increases the formation of energy-dependent ion-carrying systems, and if the transport system is deprived of an energy source, then selective transport capacity could be lost with the membrane becoming freely permeable to these ions. Since water control cells and cells treated with GA for 6 hr or less do not release their ions (although extraction data show that these ions are present), it follows that the ion-carrier system must be present for the inhibitors of oxidative phosphorylation to be effective in causing rapid ion efflux (Fig. 3).

Cycloheximide is an effective inhibitor of ion release in aleurone layers even when administered after ion release has begun (Fig. 7). Thus, when cycloheximide is given 9 hr after

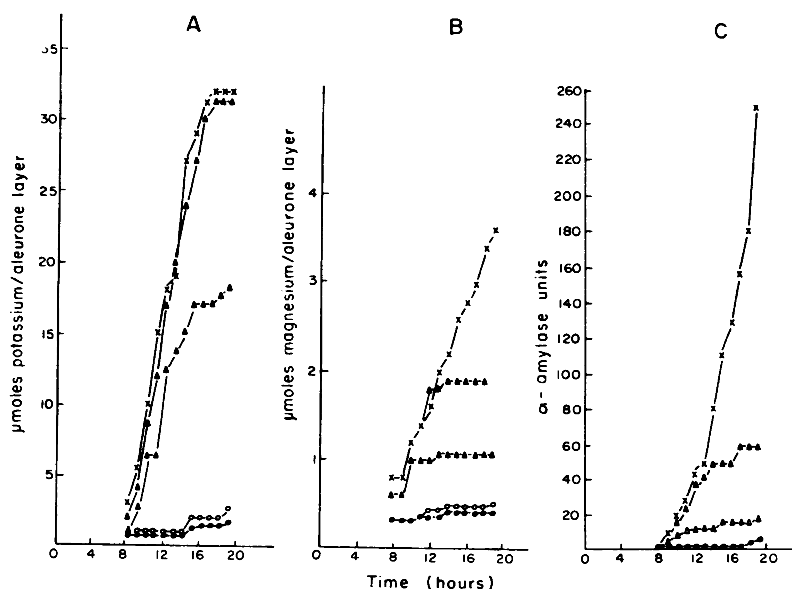

FIG. 7. Effect of 10 μg/ml cycloheximide on potassium (A), magnesium (B), and α-amylase (C) release from 50 aleurone layers incubated in 25 ml of medium. ●: H_2O control and inhibitor added with GA at time 0; ×: GA control; ○: inhibitor given 4 hr after GA; △: inhibitor given 9 hr after GA; ▲: inhibitor given 12 hr after GA.

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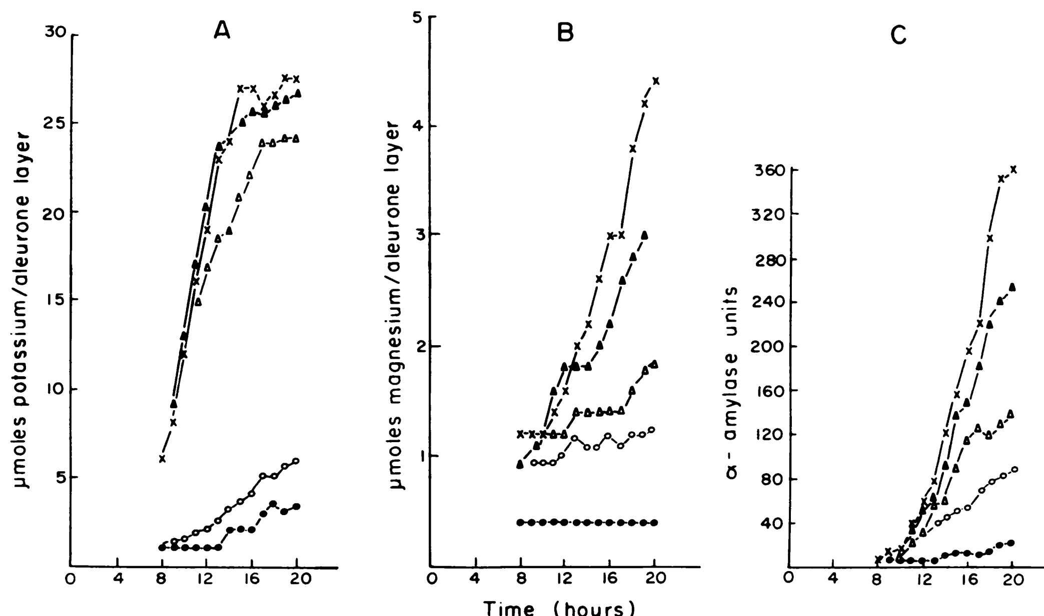

FIG. 8. As Fig. 7, except that 5 mM 6-methylpurine was added with GA at 5, 9, and 12 hr after GA treatment.

GA, potassium release is prevented, suggesting that for the continued release of this ion, protein synthesis is required (Fig. 7). Methylpurine is an effective inhibitor of ion release, especially when given with GA, but it also effectively inhibits magnesium release when given at 9 and 12 hr after GA (Fig. 8). Its effect on potassium release is less marked and probably reflects the fact that the time course of potassium release is shorter than that of magnesium (Fig. 8). Thus, 6-methylpurine given 12 hr after GA has an effective inhibition period of only 4 hr for potassium but at least 8 hr for magnesium (Fig. 8). It cannot be argued that inhibition of ion release by cycloheximide and 6-methylpurine during the phase of active efflux is a result of a reduction in the level of available ions, since experiments with inhibitors of oxidative phosphorylation indicate clearly that ions which potentially can be released are present in these cells (Figs. 3, 4, 7, and 8).

The data presented in this paper are consistent with the hypothesis that GA brings about the formation of an energy-linked ion-carrying system in aleurone cells. The formation and maintenance of this system is dependent on RNA and protein synthesis. The functioning of the system requires the continuous supply of energy, probably in the form of ATP. When deprived of an energy supply, the ion-carrying system becomes inoperative and allows for the loss of ions from the cells so that these ions reach equilibrium with the outside medium.

The significance of this GA-controlled ion efflux may extend beyond its role in mineral redistribution. Varner and Mense (26) have shown that movement of α-amylase through the cell walls of aleurone tissue is dependent on the presence of cations. They speculate that the rate of ion release functions to regulate α-amylase release into the endosperm (26).

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