Plant Physiol. (1982) 70, 1149–1155
0032-0889/82/70/1149/07/$00.50/0

# α-Amylase Secretion by Single Barley Aleurone Layers[1]

Received for publication December 3, 1981 and in revised form April 29, 1982

BENJAMIN A. MOLL AND RUSSELL L. JONES[2]
*Department of Botany, University of California, Berkeley, California 94720*

## ABSTRACT

**The secretion of α-amylase from single isolated (*Hordeum vulgare* L. cv Himalaya) aleurone layers was studied in an automated flow-through apparatus. The apparatus, consisting of a modified sample analyzer linked to a chart recorder, automatically samples the flow-through medium at 1 minute intervals and assays for the presence of α-amylase. The release of α-amylase from aleurone layers begins after 5 to 6 hours of exposure to gibberellic acid and reaches a maximum rate after 10 to 12 hours. The release of α-amylase shows a marked dependence on $Ca^{2+}$, and in the absence of $Ca^{2+}$ it is only 20% of that in the presence of 10 millimolar $Ca^{2+}$. Withdrawal of $Ca^{2+}$ from the flow-through medium results in the immediate cessation of enzyme release and addition of $Ca^{2+}$ causes immediate resumption of the release process. The effect of $Ca^{2+}$ is concentration-dependent, being half-maximal at 1 millimolar $Ca^{2+}$ and saturated at 10 millimolar $Ca^{2+}$. Ruthenium red, which blocks $Ca^{2+}$ but not $Mg^{2+}$ efflux from barley aleurone layers, renders α-amylase release insensitive to $Ca^{2+}$ withdrawal. Inhibitors of respiratory metabolism cause a burst of α-amylase release which lasts for 0.5 to 5 hours. Following this phase of enhanced α-amylase release, the rate of release declines to zero. Pretreatment of aleurone layers with HCl prior to incubation in HCN also causes a burst of α-amylase release, indicating that the inhibitor is affecting the secretion of α-amylase and not its movement through the cell wall. The rapid inhibition of α-amylase release upon incubation of aleurone layers at low temperature (5°C) or in 0.5 molar mannitol also indicates that enzyme release is dependent on a metabolically linked process and is not diffusion-limited. This conclusion is supported by cytochemical observations which show that, although the cell wall matrix of aleurone layers undergoes extensive digestion after gibberellin treatment, the innermost part of the cell wall is not degraded and could influence enzyme release.**

A characteristic of the aleurone layer of cereal seeds is its capacity to secrete a wide spectrum of hydrolytic enzymes, which play a role in endosperm degradation during germination. In barley aleurone, the synthesis of many of these enzymes is controlled by GA[3] produced by the embryo (17). Little is known, however, about the control of enzyme release or the cellular mechanism of protein transport. The release of hydrolytic enzymes from aleurone cells requires transport of the enzyme across the plasmalemma and movement through the matrix of the highly thickened cell wall.

Varner and Mense (18) examined α-amylase release from single aleurone layers of barley in a flow-through device and concluded that enzyme release into the incubation medium could be resolved into two parts, secretion and release. The process of enzyme secretion, which they defined as transport of completed α-amylase across the plasma membrane, was an energy-dependent process, whereas release of the completed molecule into the incubation medium was diffusion-limited (18). Although movement of α-amylase through the cell wall was by diffusion, these authors suggested that this process could be regulated by the presence of ions. The hypothesis that ions could regulate α-amylase diffusion through the cell wall received support from the work of Jones (10), who showed that the release of $K^+$, $Mg^{2+}$, and $PO_4^{3-}$ from the aleurone layer was dependent on the presence of $GA_3$. By controlling the release of ions, therefore, GA could exert control over the release of α-amylase as Varner and Mense (18) proposed.

A role for the cell wall in the regulation of enzyme release from the aleurone layer is predicated on the assumption that the cell wall is an impermeable barrier to these proteins. Ashford and Jacobsen (2) have shown that the release of acid phosphatase from aleurone layers occurs only after cell wall digestion. They showed that acid phosphatase accumulated between the plasma membrane and the cell wall during incubation in $H_2O$ and that during incubation in GA this enzyme then moved into the incubation medium following channels that appeared during digestion of the cell wall. Furthermore, after 24 h in $GA_3$, acid phosphatase could not be detected in the digested regions of the cell wall, indicating that at this stage the wall did not offer a barrier to the release of this enzyme (2). Because Varner and Mense (18) conducted their experiments on enzyme release on aleurone layers incubated in $GA_3$ for 24 h, it is difficult to compare their observations and conclusions concerning the role of the cell wall in regulating enzyme release with those of Ashford and Jacobsen (2) on cell wall degradation and the movement of an acid phosphatase through the cell wall.

We have reinvestigated the phenomenon of enzyme release from barley aleurone layers using an automated flow-through apparatus which can measure the α-amylase output of a single aleurone layer at intervals of 1 min. We have coupled our study of the kinetics of enzyme release to cytochemical investigations of cell wall structure at the electron microscope level.

## MATERIALS AND METHODS

**Preparation of Plant Material.** Seeds of barley (*Hordeum vulgare* L. cv Himalaya) were deembryonated and endosperm halves were surface sterilized as described by Chrispeels and Varner (4). Sterilized seeds were imbibed on sterile sand moistened with sterile distilled $H_2O$, and aleurone layers were dissected from the starchy endosperm after 3 to 4 d imbibition (4).

**Flow-Through Apparatus.** α-Amylase production by single aleurone layers was monitored in an automated flow-through apparatus (Fig. 1). The apparatus consisted of a timer which controlled a series of gang valves which operated air-driven pipettes and a spectrophotometer, all salvaged from a Gilson Automatic Sample Analyzer model 4000 (Gilson Medical Electronics, Middleton, WI). These components were modified by the addition of a chamber to hold a single aleurone layer and temperature-regulated

[1] Supported by National Science Foundation Grant PCM 13286 to R. L. J.

[2] To whom reprint requests should be addressed.

[3] Abbreviations: GA, gibberellin(s); DNP, 2,4-dinitrophenol; CCCP, carbonyl cyanide-*m*-chlorophenyl-hydrazone.

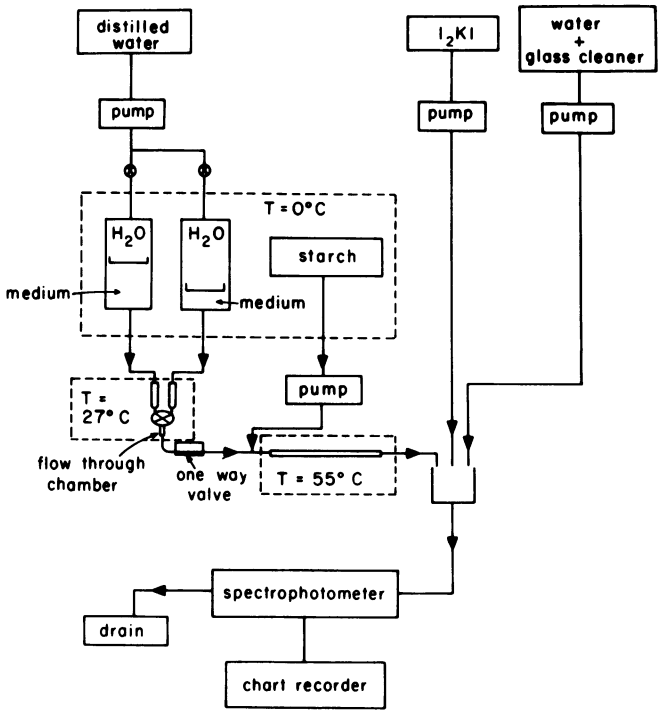


FIG. 1. Diagram of the automatic flow-through apparatus. An aleurone is placed in the flow-through chamber, and medium flows from the selected reservoir. Pumping is by air-driven precision pipetters controlled by a timer. Medium from the chamber is mixed with starch and incubated at 55°C. The starch enzyme mixture is then mixed with $I_2KI$ solution and the resultant O.D. measured and automatically recorded.

baths for sample chamber, media, and assay line, and the replacement of the electronic components of the spectrophotometer with an integrated circuit and field effect transistor-based log amplifier. The output of the spectrophotometer was recorded on a Linear Instruments chart recorder (model 255; Linear Instruments, Irvine, CA). A detailed description of the construction and operation of the flow-through apparatus and assay procedure is available in Moll (14).

**Incubation.** Except for the experiment shown in Figure 2, prior to emplacement in the flow-through sample chamber, aleurone layers were pretreated with $GA_3$ for 12 to 15 h in 25-ml flasks in 2 ml standard medium consisting of 10 mM $CaCl_2$, 1 mM sodium acetate (pH 4.8), and 1 to 10 μM $GA_3$ (Sigma) (4).

Aleurone layers incubated in the flow-through apparatus were exposed to the standard flow-through medium containing 1 to 10 μM $GA_3$, 1 mM $KH_2PO_4$, 200 μM EDTA, 50 μM $FeSO_4$, 10 mM $CaCl_2$ (except as noted), 0.01% (v/v) Triton X-100 and 2 μM chloramphenicol and adjusted to pH 5.0 with KOH. EDTA (200 μM) was added to the medium to ensure that adjustment of divalent cation concentrations would result in the desired shift in cation levels even in the presence of cation release. Inasmuch as Goodwin and Carr (8) have shown that aleurone layers of barley do not produce appreciable α-amylase in the presence of chelators unless iron is included in the medium, $FeSO_4$ (50 μM) was added. A detergent (Triton X-100, Rohm and Hass) and an antibiotic (chloramphenicol) were added to the incubation medium to reduce microbial contamination; there were no observable effects of either compound on α-amylase production by aleurone layers. Microscopic examination of media and aleurone layers at the end of each experiment revealed no bacterial contamination. In some experiments, the medium also included 2 mM DNP, 0.6 mM $NaN_3$, 1 mM HCN, or 10 μg/ml CCCP (all from Sigma), or aleurone layers were exposed to a pulse of ruthenium red (Sigma) as described in the legend for Figure 5. To establish that components of the medium were not interfering with the starch-iodine assay for α-amylase, the incubation medium was tested with the starch and $I_2KI$ reagents prior to introduction of the aleurone layer. The flow-through medium was aerated by bubbling with compressed air and was cooled to 0°C and maintained at this temperature except in the sample chamber, where the temperature was regulated at 27°C.

The flow-through medium was cycled through the sample chamber at 1 to 2 min intervals. During each cycle, 100 μl of medium was pumped past the aleurone layer. Inasmuch as the volume of the flow-through chamber was approximately 50 μl greater than that of the aleurone layer, complete replacement of the medium was ensured.

**Enzyme Assay.** α-Amylase was automatically assayed by the flow-through apparatus using a modification of the standard starch-iodine procedure (4). To increase the sensitivity, assays were performed at 55°C for 10 to 20 min (depending on the flow rate of the system), using starch, and subsequently $I_2KI$, at 4 times the standard concentrations. The starch was maintained at 0°C prior to use. To allow prolonged starch digestion, the assay line had a volume sufficient to hold 10 samples, that is, 10 cycles. Inasmuch as there was no sample-to-sample separation in the assay line, mixing could affect the time resolution of the flow-through experiments. When a sample of barley α-amylase was introduced into the sample chamber without an aleurone layer, a square-wave output of enzyme activity was observed, indicating that assay conditions did not limit the time resolution.

Data for secretion of α-amylase are presented as α-amylase release rate in relative units, one unit being approximately equal to one standard α-amylase unit per aleurone per h. All experiments reported in this paper were repeated at least 4 times, and the data presented are representative of the results obtained.

**Electron Microscopy.** Cell wall polysaccharides were localized in GA-treated aleurone layers by the periodic acid-silver methenamine technique (15). Ultra-thin sections of glutaraldehyde-fixed aleurone layers (12) were mounted on gold grids, oxidized for 20 min on 1% (w/v) periodic acid and stained with silver methenamine for two periods of 30 min each. Stained sections were washed in 5% (w/v) sodium thiosulfate for 5 min followed by rinsing in $H_2O$ and examination in an electron microscope.

## RESULTS

**Incubation Conditions.** Incubation of $GA_3$-pretreated aleurone layers in a flow-through medium containing only $CaCl_2$ (10 mM) and $GA_3$ (1–10 μM) as in the standard flask medium resulted in a gradual decline in the output of α-amylase. Addition of 1 mM $KH_2PO_4$ to the flow-through medium, however, increased α-am-

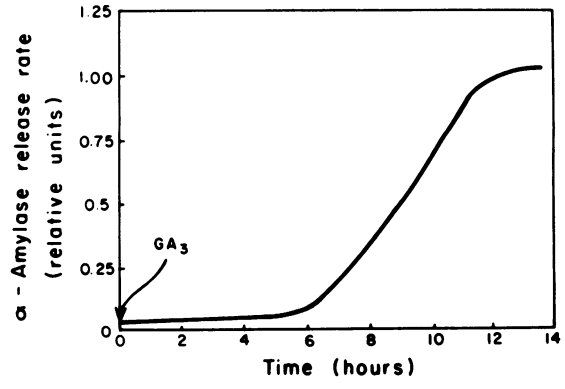


FIG. 2. The induction of α-amylase release by a single aleurone in the flow-through apparatus. $GA_3$ (10 μM) was added at time 0 in the standard flow-through medium. The aleurone was not preincubated in $GA_3$ medium.

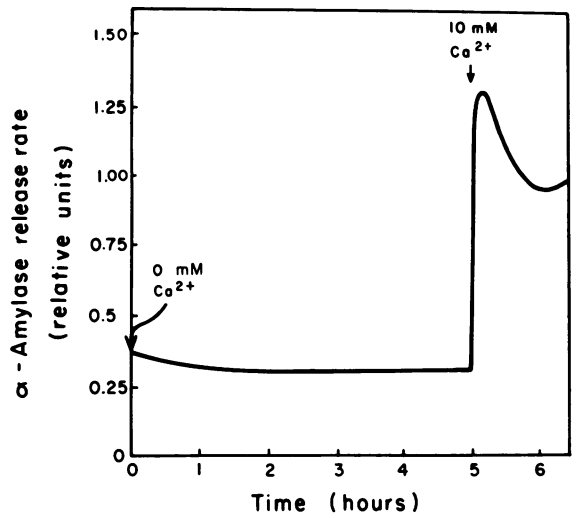


FIG. 3. The long-term, low-$Ca^{2+}$ inhibition of α-amylase release by a single aleurone and the rapid response to high $Ca^{2+}$ as measured in the flow-through apparatus.

ylase output and resulted in the production of enzyme at a rate comparable to that observed by using conventional methods of aleurone incubation (4, 10). Therefore, $KH_2PO_4$ (1 mM) was included in all flow-through experiments.

**$GA_3$ Promotion of α-Amylase Release.** The time course of $GA_3$-enhanced α-amylase release in the flow-through apparatus is shown in Figure 2. There was a lag of 5 to 6 h before the rate of release into the medium changed, and the maximum rate of α-amylase release was not achieved until after 10 to 12 h of incubation. The effect of $GA_3$ was to stimulate the rate of α-amylase release by a factor of 20 to 30 (Fig. 2). Although it is clear that the enhancement of α-amylase synthesis and release by $GA_3$ can be demonstrated in the flow-through apparatus, for convenience aleurones were usually pretreated in $GA_3$ for 12 to 15 h under standard incubation conditions in 25-ml Erlenmeyer flasks prior to their introduction into the flow-through apparatus.

**Effects of $Ca^{2+}$ and Ruthenium Red on Amylase Release.** The release of α-amylase from GA-treated aleurone layers shows a marked dependence on $Ca^{2+}$. In the absence of $Ca^{2+}$, the rate of α-amylase release was 20% to 30% of the rate observed in the presence of 10 mM $Ca^{2+}$ (Figs. 3 and 4). The effect of $Ca^{2+}$ was not transient, although withdrawal of the cation from the medium resulted in inhibition which lasted for the duration of the withdrawal period (Fig. 3). Recovery of the rate of α-amylase release following addition of $Ca^{2+}$ was rapid even after an extended period of withdrawal (Fig. 3). Both the recovery of α-amylase release from the effect of $Ca^{2+}$ withdrawal and the onset of the inhibitory effects of $Ca^{2+}$ had half-times on the order of 10 min (Figs. 3 and 4).

The rate of α-amylase release from $GA_3$-treated aleurone layers incubated in the flow-through apparatus varied according to the $Ca^{2+}$ concentration (Fig. 4). The response to $Ca^{2+}$ was half-maximal at 1 mM and saturated at 10 mM (Fig. 4 and unreported data). The responsiveness of an aleurone layer to $Ca^{2+}$ addition and withdrawal was routinely used as a measure of the capacity of the aleurone layer to produce α-amylase at a constant rate for 8 to 12 h. To accomplish this, aleurone layers were introduced into the flow-through apparatus and allowed to secrete α-amylase in the presence of 5 or 10 mM $CaCl_2$ for 1 to 2 h. After a shift to medium with no $Ca^{2+}$, the layers were returned to high $Ca^{2+}$ and the rate of α-amylase release was monitored. Only those layers showing recovery of maximal α-amylase release rates after the $Ca^{2+}$ shift were selected for further experimentation (Figs. 4–7).

Ruthenium red is known to block $Ca^{2+}$ transport (9, 19), and

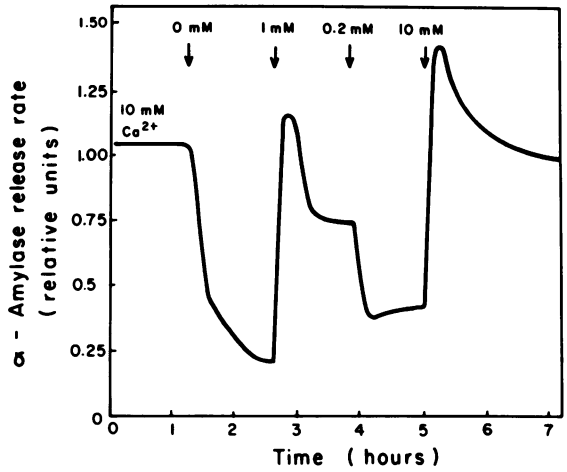


FIG. 4. The response of an aleurone to different concentrations of $Ca^{2+}$ as measured in the flow-through apparatus.

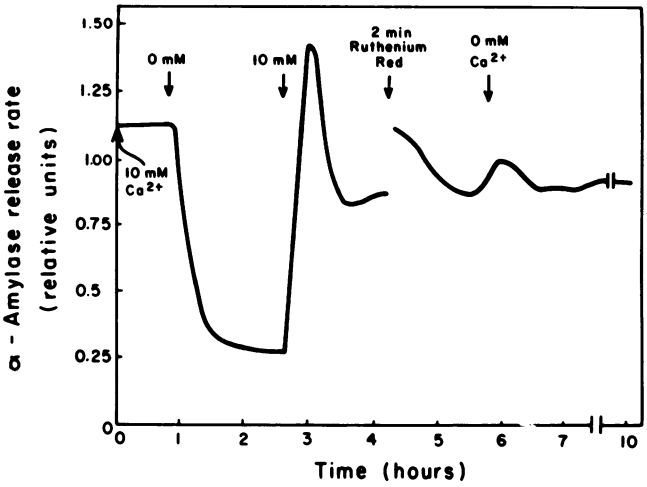


FIG. 5. The effect of ruthenium red on α-amylase release by an aleurone in the flow-through apparatus. The sensitivity of α-amylase release to $Ca^{2+}$ was verified by a switch to 0 $Ca^{2+}$ and return to high $Ca^{2+}$. The aleurone layer was exposed to a 1 to 2 min pulse of ruthenium red (5 mg $ml^{-1}$) by immersion of the flow-through chamber in this solution. A subsequent switch to 0 $Ca^{2+}$ did not result in a decrease in α-amylase release rate.

we investigated the effect of this compound on $Ca^{2+}$ efflux and $Ca^{2+}$-stimulated α-amylase release by barley aleurone layers. That ruthenium red inhibits $Ca^{2+}$ efflux from GA-treated aleurone layers is shown by the following experiment. Aleurone layers (100) were incubated in flasks in 1 μM $GA_3$ and 10 mM $Ca^{2+}$ for 12 h then transferred to medium without $Ca^{2+}$ for 3.5 h. Samples of the medium (1 ml) were withdrawn at 30-min intervals and diluted with 1 ml 1 N HCl containing 0.1 M $MnCl_2$, and the $Ca^{2+}$ and $Mg^{2+}$ contents were measured in an Atomic Absorption Spectrophotometer (model 290; Perkin Elmer, Mountain View, CA). After seven consecutive samples, aleurone layers were exposed to ruthenium red (200 μg $ml^{-1}$) for 5 min, washed briefly with $H_2O$ and placed in $Ca^{2+}$-free medium. The medium was sampled at 30 min intervals for 2 h. Ruthenium red reduced $Ca^{2+}$ release by the aleurone layers by about 60%, but it had no effect on the efflux of $Mg^{2+}$ from this tissue (data not shown). The effect of ruthenium red on α-amylase release by a single aleurone layer in a flow-through experiment is shown in Figure 5. The data show that following exposure to ruthenium red, α-amylase release was unaffected by a switch of the flow-through medium from 10 mM to 0 mM $Ca^{2+}$.

**Effects of Metabolic Inhibitors and Low Temperature.** Sodium

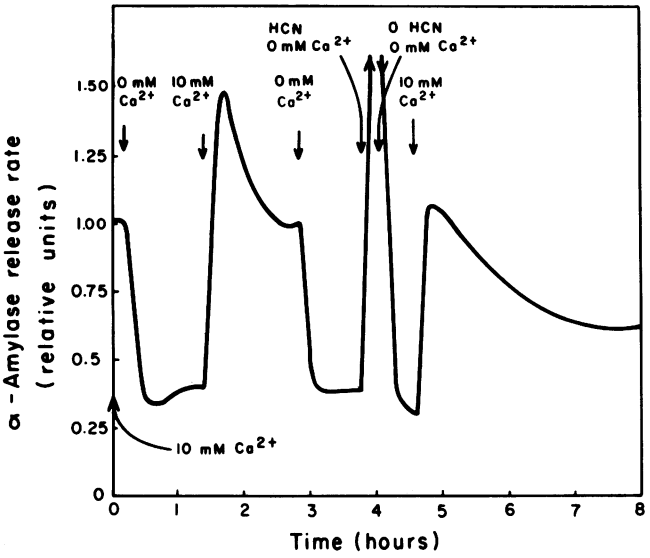


FIG. 6. The response of an aleurone layer to a 15-min pulse of 1 mM HCN in the flow-through apparatus following determination of low and high $Ca^{2+}$ rates. The release spike was off scale on the recorder.

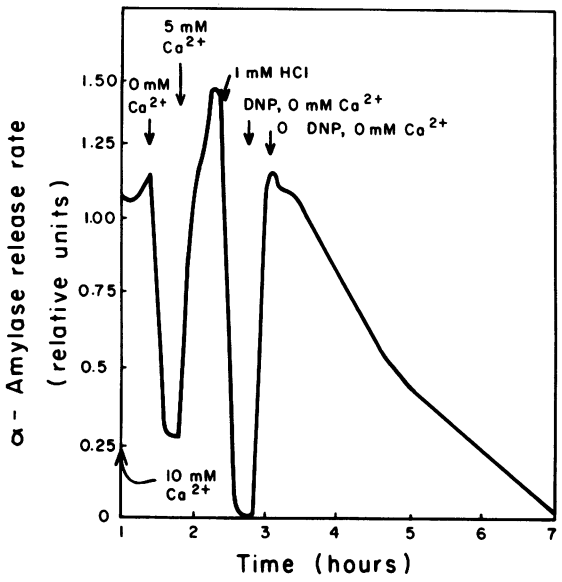


FIG. 7. The response of an aleurone layer to 2 mM DNP in the flow-through apparatus. Following determination of responsiveness to $Ca^{2+}$, the aleurone was treated with 1 mM HCl.

azide, HCN, DNP, and CCCP were used to determine the secretory response of the aleurone layer to inhibitors of respiratory metabolism. In all cases, addition of these inhibitors resulted in an increase in the rate of α-amylase release above that found with 10 mM $Ca^{2+}$. This burst of α-amylase release lasted for 0.5 to 5 h and was followed by a decline in enzyme release reaching zero activity after several hours. When aleurone layers were exposed to a 15-min pulse of 1 mM HCN following establishment of a low secretory rate in the absence of $Ca^{2+}$, there was a characteristic spike of α-amylase release reaching a level considerably above the high $Ca^{2+}$ rate of α-amylase release (Fig. 6). When the medium was maintained at 0 $Ca^{2+}$ following the HCN pulse, the rate of α-amylase release declined to the rate established prior to HCN treatment (Fig. 6), and a subsequent shift in the $Ca^{2+}$ concentration to 10 mM resulted in elevation of the rate of α-amylase release.

In an effort to determine whether the α-amylase released from aleurone layers in response to metabolic inhibitors came from a cytoplasmic compartment or from the cell wall, aleurone layers

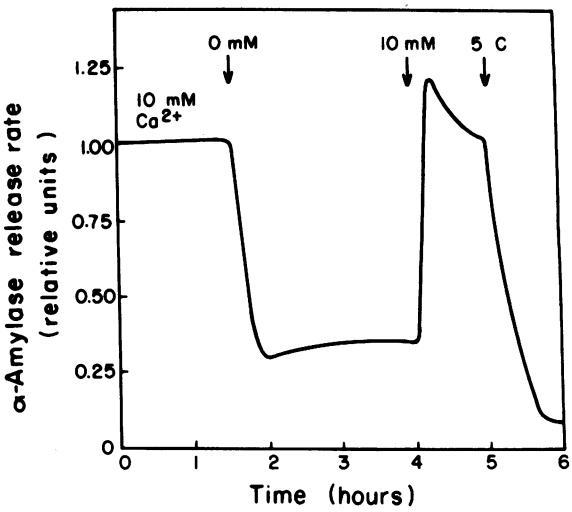


FIG. 8. The effect of low temperature on α-amylase release from an aleurone in the flow-through apparatus. Following determination of low and high $Ca^{2+}$ steady-state rates, the flow-through chamber was immersed in a 5°C bath.

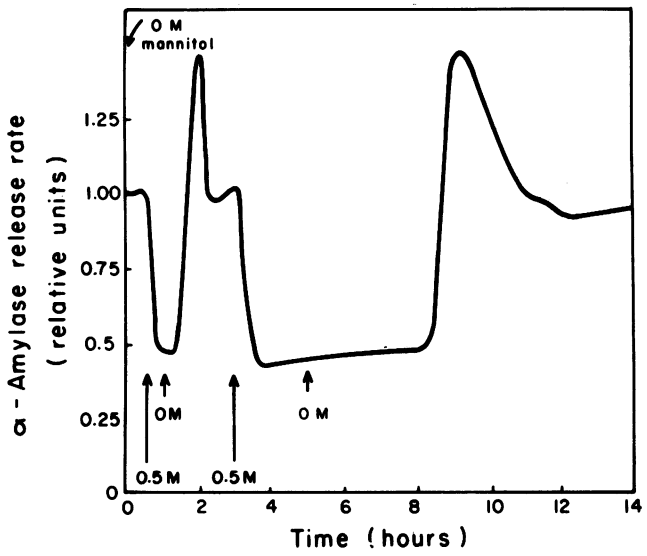


FIG. 9. Rapid inhibition of α-amylase release from an aleurone incubated in 10 mM $Ca^{2+}$ in the flow-through apparatus by 0.5 M mannitol and its reversal by return to 0 mannitol.

were exposed to 1 mM HCl to denature extra-cytoplasmic α-amylase (18) prior to exposure to DNP (Fig. 7). After demonstrating the $Ca^{2+}$ sensitivity of the layer, the medium was then switched to one containing DNP without $Ca^{2+}$, resulting in a characteristic spike of α-amylase release (Fig. 7). Replacement of DNP with a medium containing no $Ca^{2+}$ caused the rate of α-amylase release to decline gradually to zero (Fig. 7).

To distinguish between the involvement of metabolism and diffusion in the release of α-amylase from the aleurone layer, enzyme release was measured at low temperature (Fig. 8). A decrease in temperature of the medium from 25°C to 5°C caused a rapid cessation of α-amylase release from the aleurone layer. This response was detected within minutes, and the rate of enzyme release decayed as rapidly as it did when $Ca^{2+}$ was withdrawn (Fig. 8). Low temperature, however, caused the enzyme release rate to fall to near zero, whereas $Ca^{2+}$ withdrawal resulted in a rate of α-amylase release which was 30% of the high $Ca^{2+}$ rate (Fig. 8).

**Effects of Osmotica.** Osmotica are known to inhibit the pro-

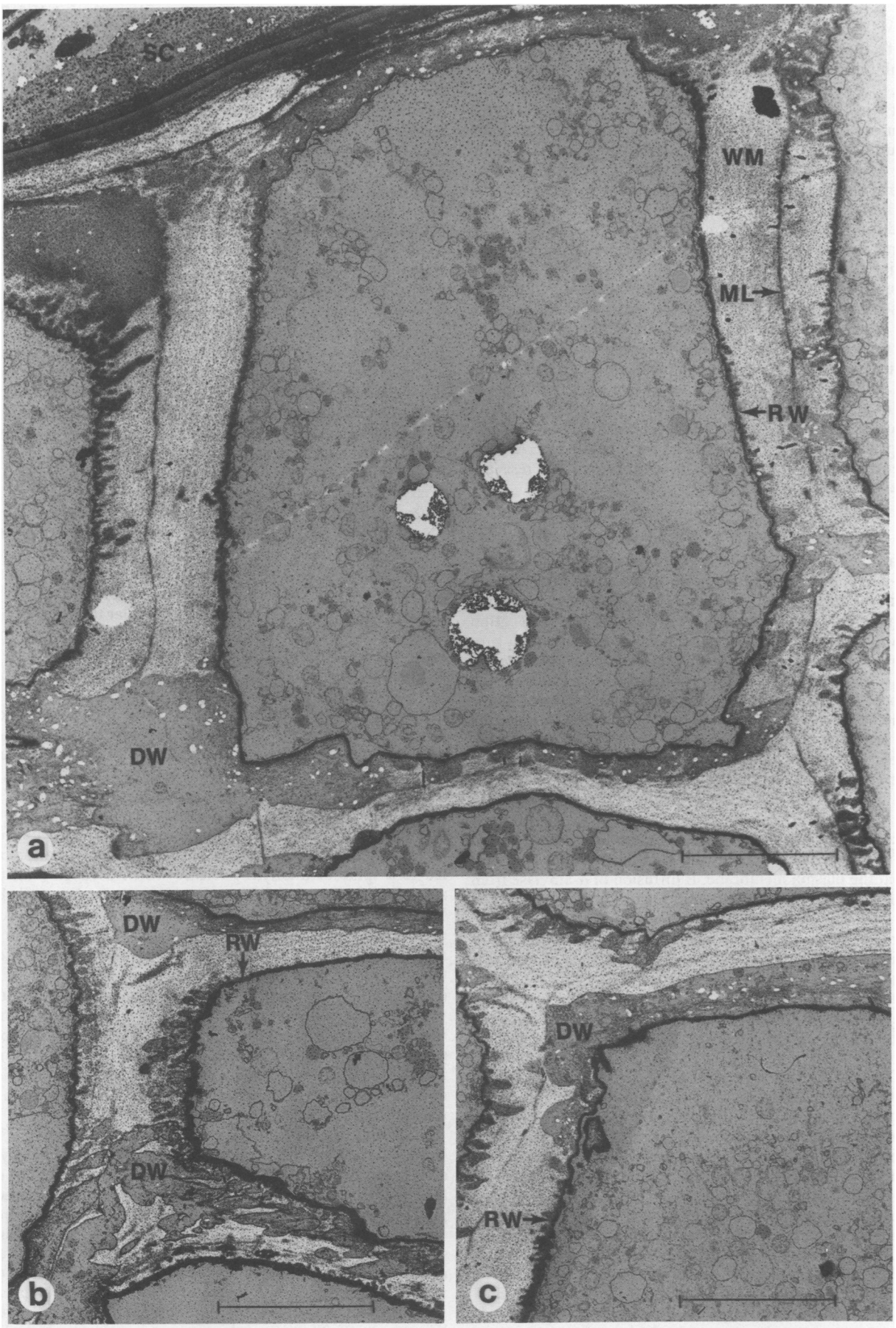


FIG. 10. Electron micrographs of periodic acid silver-methenamine stained sections of aleurone tissue after 18 h incubation in 10 $\mu$M $GA_3$ showing digestion of the cell wall matrix. DW, digested areas of cell wall; ML, middle lamella; SC, seed coat; RW resistant wall; WM, wall matrix. All × 54,000. Bars all represent 0.5 $\mu$m.

duction of α-amylase by barley aleurone layers (1, 3, 11). Mannitol (0.5 M) inhibited α-amylase release within minutes after application (Fig. 9). Recovery of the rate of α-amylase release following treatment with mannitol had two interesting features. First, the time from osmotic inhibition to the onset of recovery depended on the duration of exposure to mannitol. Thus, after a 15-min exposure to mannitol, recovery was initiated within 10 min, whereas after a 1-h exposure to mannitol, recovery did not begin until 90 min after removal of the mannitol solution (Fig. 9). Second, during recovery from osmotic inhibition, the rate of α-amylase release showed a large overshoot before returning to the normal high $Ca^{2+}$ level.

**Electron Microscopy.** Incubation of aleurone layers in GA for 18 h caused pronounced changes in cell wall structure (Fig. 10). With the exception of the innermost part of the cell wall, the resistant wall (16), there is considerable cell wall breakdown resulting in the formation of wide channels in the cell wall matrix (Fig. 10). Wall breakdown can be extensive and result in the digestion of all of the cell wall lying between the resistant walls of adjacent cells. There is no evidence that the resistant wall breaks down (Fig. 10). Indeed, even after 72 h of incubation in GA when cell contents and cell wall matrix have disappeared, the resistant wall remains (not shown).

## DISCUSSION

Using a flow-through apparatus to monitor α-amylase production at 1-min intervals from aleurone layers of barley we have shown that the release of this enzyme is dependent on metabolic level and temperature and can be controlled by the divalent cation concentration of the surrounding medium. Our kinetic data support the hypothesis that when a steady rate of α-amylase secretion has been established after 12 to 14 h of GA treatment, the regulation of enzyme transport from this tissue is exerted at the plasma membrane and not by limitation of diffusion through the cell wall, as proposed by Varner and Mense (18). Our electron microscopic observations, however, point to a resistant cell wall which apparently remains intact even after prolonged $GA_3$ treatment and could thus play some role in the control of α-amylase release.

Our experiments with $Ca^{2+}$, low temperature and mannitol allow us to distinguish between diffusion-limited processes and transport at the plasma membrane. If diffusion of α-amylase from a large cell wall pool were the rate-limiting process controlling the rate of α-amylase release, we would expect low temperature and osmoticum to have very little immediate effect on the rate of enzyme release. Our results clearly show that effects of temperature and osmotica are rapid and dramatic (Figs. 8 and 9) and are unlikely to be mediated via effects on diffusion from a cell wall pool of α-amylase. Rather, these results are consistent with the hypothesis that the rate of α-amylase release is set at the plasma membrane.

The effect of $Ca^{2+}$ in increasing α-amylase release (Figs. 3 and 4) could be interpreted as an ion exchange phenomenon in the cell wall rather than an effect on secretion at the plasma membrane. Were this the case, we would expect $Ca^{2+}$ to have transient effects, permitting diffusion out of the cell wall in its presence, or loading of the wall pool in its absence. Neither the former (Fig. 4) nor the latter (B. Moll, unpublished) was the case. Inasmuch as $Ca^{2+}$ clearly affects steady-state rates as well as transient ones, it must be affecting more than the mobility of the α-amylase in the cell wall. The specificity of the secretory response to $Ca^{2+}$ is also illustrated by the effect of other cations on this process. $Ca^{2+}$ can be substituted in this role by $Sr^{2+}$ and to a lesser degree by $Ba^{2+}$ and $Mg^{2+}$, whereas other divalent and monovalent ions tested do not promote α-amylase release (unpublished data).

Experiments with ruthenium red also provide convincing evidence that the effects of $Ca^{2+}$ are on the secretory process. This compound has been shown to be a potent inhibitor of $Ca^{2+}$ uptake and efflux by animal cells and mitochondria (9, 19). Ruthenium red also interferes with $Ca^{2+}$ efflux from aleurone layers of barley, although it is without effect on the release of $Mg^{2+}$ from this tissue. Following exposure to ruthenium red, the secretion of α-amylase from aleurone layers is independent of the presence of $Ca^{2+}$ (Fig. 5). Based on the known mechanism of ruthenium red action in animal and plant cells, the most likely explanation of its effect on α-amylase release involves its role in the regulation of cytoplasmic $Ca^{2+}$ concentration. The control of the secretion of α-amylase by the regulation of $Ca^{2+}$ levels has been demonstrated in parotid exocrine cells (13). Agents which stimulate $Ca^{2+}$ uptake by the cell or inhibit $Ca^{2+}$ sequestration by mitochondria promote α-amylase release (13). By inhibiting $Ca^{2+}$ release at the plasmalemma, or preventing $Ca^{2+}$ uptake by mitochondria, ruthenium red could maintain high cytoplasmic levels of $Ca^{2+}$ even when the external $Ca^{2+}$ supply is withdrawn (as in our experiment shown in Fig. 5). It is clear that the maintenance of high levels of α-amylase release which accompanies $Ca^{2+}$ withdrawal in ruthenium red-treated tissues cannot be attributed to the leaching of the enzyme from the cell wall by cations. The dependence of α-amylase release by aleurone cells on $Ca^{2+}$ is reminiscent of the control of release of a wide spectrum of secretory products in animals. It is widely accepted that $Ca^{2+}$ plays a central role in stimulus-secretion coupling and that the process of exocytosis is directly dependent on $Ca^{2+}$ (6, 7, 13).

Our experiments with uncouplers and inhibitors of oxidative metabolism also indicate membrane regulation of α-amylase release (Figs. 6 and 7). These data are similar to those obtained by Varner and Mense (18), who also demonstrated that treatment of aleurone layers with DNP resulted in an increase in the rate of α-amylase release (see Figs. 2 and 4 of Ref. 18). The inability of DNP to inhibit the release of α-amylase was interpreted as evidence favoring the presence of a pool of enzyme which was diffusing through the wall. It was also argued that the wall pool of α-amylase should be readily denatured by 1 mM HCl and that if HCl treatment preceded the addition of DNP, there should be no release of α-amylase from the aleurone layer. Neither Varner and Mense (Fig. 4 of Ref. 18) nor we found this to be so experimentally. Even when the putative cell wall pool of α-amylase was denatured by 1 mM HCl, subsequent incubation in DNP led to a spike of α-amylase release which decayed with time to zero (Fig. 7). One possible explanation of our data and those of Varner and Mense (18) would be that the transport of α-amylase across the plasmalemma has no immediate dependence on energy metabolism. Consequently, inhibition of metabolism would favor the movement of α-amylase from a pool inside the cell to the outside. It is of interest to note in this context that changes in respiratory metabolism also affect the release of ions from aleurone layers. The addition of uncouplers or inhibitors of respiration to barley aleurone layers results in a rapid efflux of ions into the incubation medium (10). The possibility that the rapid release of ions in response to inhibitor treatments promotes α-amylase release from the cell wall cannot be overlooked.

Following the addition of $Ca^{2+}$ after a period of $Ca^{2+}$ withdrawal, the aleurone in the flow-through apparatus typically shows a transient overshoot in the rate of α-amylase secretion, usually lasting 15 to 20 min. These kinetics suggest a small presecretory pool of α-amylase which does not respond to stimuli as rapidly as does secretion. Thus, when a stimulus which results in inhibition of α-amylase release is given, the effect on secretion is rapid as measured by the immediate cessation of α-amylase release, whereas loading of a presecretory pool continues for a time proportional to the size of the subsequent recovery overshoot (Figs. 3 and 9). The size of this proposed presecretory pool is only a fraction of the total internal α-amylase pool, which is large enough to maintain α-amylase secretion at the maximum rate for

3 to 4 h (14, 18). Treatment with cycloheximide also shows that a large pool of synthesized α-amylase exists in the aleurone layer. Our data (14) and those of Varner and Mense (18) show that cycloheximide does not affect secretion of α-amylase until after several hours of exposure. Inasmuch as cycloheximide strongly inhibits protein synthesis in the aleurone layer (5), it follows that the aleurone cells possess a pool of α-amylase the release of which is independent of new protein synthesis.

Our kinetic evidence supports the idea that under our experimental conditions regulation of α-amylase release is largely through control of a secretory process, not modification of diffusion kinetics. Cytologic evidence of considerable wall breakdown also supports this view, although it should be noted that the resistant wall does not undergo digestion (Fig. 10) and could be a site for a diffusion-limited step if one exists.

In spite of our observations of the existence of a resistant cell wall even after prolonged incubation in $GA_3$, we must conclude that our experimental evidence supports the hypothesis that α-amylase release from the barley aleurone is controlled principally by the plasma membrane not the cell wall. We have shown that release of α-amylase from aleurone layers in the flow-through apparatus is inhibited by low temperature and inhibitors of respiratory metabolism and that it is strongly dependent on the level of $Ca^{2+}$ in the incubation medium.

*Acknowledgments*—Carol Moll and Eleanor Crump are thanked for their assistance in preparing the manuscript.

## LITERATURE CITED

1. Armstrong JE, RL Jones 1973 Osmotic regulation of α-amylase synthesis and polyribosome formation in aleurone cells of barley. J Cell Biol 59: 444–455
2. Ashford AE, JV Jacobsen 1974 Cytochemical localization of phosphatase in barley aleurone cells: the pathway of gibberellic-acid-induced enzyme release. Planta 120: 81–105
3. Chrispeels MJ 1973 Mechanism of osmotic regulation of hydrolase synthesis in aleurone cells of barley: Inhibition of protein synthesis. Biochem Biophys Res Commun 53: 99–104
4. Chrispeels MJ, JE Varner 1967 Gibberellic acid enhanced synthesis and release of α-amylase and ribonuclease by isolated barley aleurone layers. Plant Physiol 42: 398–406
5. Chrispeels MJ, JE Varner 1967 Hormonal control of enzyme synthesis: on the mode of action of gibberellic acid and abscisin in aleurone layers of barley. Plant Physiol 42: 1008–1016
6. Douglas WN 1974 Involvement of calcium in exocytosis and the exocytosis-vesiculation sequence. Biochem Soc Symp 39: 1–28
7. Foreman JC, LG Garland, JL Mongar 1976 The role of calcium in secretory processes: Model studies in mast cells. Symp Soc Exp Biol 30: 193–218
8. Goodwin PB, DJ Carr 1970 Ferric ion requirement for α-amylase synthesis by barley aleurone layers in response to gibberellic acid. Cytobios 2: 165–174
9. Hinds TR, BU Raess, FF Vincenzi 1981 Plasma membrane $Ca^{2+}$ transport: antagonism by several potential inhibitors. J Membr Biol 58: 57–65
10. Jones RL 1973 Gibberellic acid and ion release from barley aleurone tissue. Plant Physiol 52: 303–308
11. Jones RL, JE Armstrong 1971 Evidence for osmotic regulation of hydrolytic enzyme production in germinating barley seeds. Plant Physiol 48: 137–142
12. Jones RL, JM Price 1970 Gibberellic acid and the fine structure of barley aleurone cells. III. Vacuolation of the aleurone cell during the phase of ribonuclease release. Planta 94: 191–202.
13. Matthews EK 1979 Calcium translocation and control mechanisms for endocrine secretion. Symp Soc Exp Biol 33: 225–249
14. Moll BA 1980 Physiological and mathematical studies on secretion of the barley aleurone. PhD Thesis. University of California, Berkeley
15. Rambourg A 1967 An improved silver methenamine technique for the detection of periodic acid-reactive complex carbohydrates with electron microscope. J Histochem Cytochem 15: 409–412
16. Taiz L, RL Jones 1973 Plasmodesmata and an associated cell wall component in barley aleurone tissue. Am J Bot 60: 67–75
17. Varner JE, DT Ho 1976 The role of hormones in the integration of seedling growth. *In* J Papaconstantinou, ed, The Molecular Biology of Hormone Action. Academic Press, New York, pp 173–194
18. Varner JE, RM Mense 1972 Characteristics of the process of enzyme release from secretory plant cells. Plant Physiol 49: 187–189
19. Watson EL, FF Vincenzi, PW Davis 1971 $Ca^{2+}$-activated membrane ATPase: selective inhibition by ruthenium red. Biochim Biophys Acta 249: 606–610