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Protoplasts isolated from aleurone layers of wild oat (*Avena fatua* L.) exhibit the classic response to gibberellic acid

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Abstract. Viable, long-lived, gibberellic acid (GA_3)-responsive protoplasts have, for the first time, been isolated from aleurone layers of mature wild oat (*Avena fatua* L.) grain. More than 90% of the cells of aleurone layers are recovered as protoplasts, and these respond to treatment with GA_3 in essentially the same manner as the tissue from which they were derived. Protoplasts become vacuolate during incubation in vitro and, although not dependent upon GA_3, vacuolation is markedly stimulated by the hormone. Amylase and ribonuclease (RNase) are produced and secreted only in the presence of GA_3 and only after lag periods of 3 d and 4 d respectively. The amounts of amylase produced and secreted are proportional to GA_3 concentrations as low as $1.61 \cdot 10^{-13}$ M. With increasing concentrations of mannitol in the culture medium both vacuolation and the GA_3-induced production and secretion of enzymes are inhibited progressively, the latter being precluded by 0.6 M to 0.7 M mannitol.

Key words: Aleurone layer – Amylase – *Avena* – Gibberellin – Protoplast – Ribonuclease.

Introduction

Isolated plant protoplasts are rapidly gaining recognition as useful research tools for plant biologists, and have been used with considerable success in a variety of physiological, biochemical and genetic investigations. Their potential for elucidating aspects of hormonally controlled events in gibberellin-sensitive plant tissues has not been overlooked. However, few definitive articles have been published. Wasilewska and Kleczkowski (1974) have demonstrated that protoplasts, isolated from apices of etiolated gibberellin-sensitive maize seedlings, exhibit altered patterns of newly synthesized nuclear RNA after treatment with gibberellic acid (GA_3). Furthermore, a similar effect upon their transcriptional activity can be induced by cyclic adenosine monophosphate (Tarantowicz-Marek and Kleczkowski 1975). Attempts at isolating GA_3-responsive protoplasts from the aleurone layer of cereal grains, probably the most thoroughly investigated gibberellin-sensitive plant tissue, have not met with the same degree of success. The aleurone cell wall has proven difficult to remove completely and at best only approximately 20% of the cells of aleurone layers have been recovered as live separated cells or protoplasts (Taiz and Jones 1971; Eastwood 1977). Furthermore, despite many attempts neither separated cells nor protoplasts have been shown to exhibit the classic response to GA_3 (Jones et al. 1973; Eastwood 1977; Taiz personal communication 1981).

Efficient isolation of viable, long-lived, GA_3-responsive protoplasts from aleurone layers of *Avena fatua* has now been achieved, permitting an exciting new approach to biochemical and physiological investigations of this plant tissue.

Abbreviations: GA_3 = gibberellic acid$_3$; RNase = ribonuclease

Materials and methods

Seeds. Mature, shedding seeds of wild oats were collected from a field near Oxford in August 1979 and stored immediately at $4 \pm 1°$ C over anhydrous $CaCl_2$. Second seeds of *A. fatua* type fA (Thurston 1957) were selected from the seed stock, dehusked and stored at $4 \pm 1°$ C over anhydrous $CaCl_2$. Isolated embryos of these grain exhibited innate dormancy.

Preparation of aleurone layers. The embryo and tip of the dry grain distal to the embryo were cut off. De-embryonated grains were cut longitudinally into 'half seeds' and weighed. 'Half seeds' were surface sterilized by incubating for 10 min at $24 \pm 1°$ C in NaOCl, 2.0% (w/v) available chlorine, with 0.2% (v/v) Teepol (Shell Chemicals, UK Ltd.), then rinsed once in sterile 0.01 M HCl and five times in sterile distilled water (Abdul-Baki 1974). All

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subsequent manipulations were performed under aseptic conditions. Media were sterilized by filtration through 0.22 μm Millipore filters (Millipore Corporation, Bedford, Massachusetts, USA). 'Half seeds' were imbibed for 20 h in the dark at 24±1° C in 50 mM L-arginine, except when stated otherwise. Imbibed 'half seeds' were incubated in the dark at 24±1° C in an isolation medium comprising, except when stated otherwise, Gamborg's B-5 medium (Gamborg et al. 1968) (Flow Laboratories Ltd, Irvine, Scotland) containing 0.4 M mannitol, 2.0% (w/v) glucose, 1.0% (w/v) Cellulase 'onozuka' R-10 (Kinki Yakult Mfg, Co., Nishinomiya, Japan), 10 μg ml^{-1} chloramphenicol, 25 units ml^{-1} nystatin, 10 mM L-arginine and 20 mM $CaCl_2$. The pH was adjusted to 5.4. Endosperm was released after 90 min by gentle shaking and the aleurone layers, with pericarp and testa attached, were washed three times in isolation medium without Cellulase 'onozuka' R-10.

Isolation and incubation of protoplasts. Freshly prepared aleurone layers were incubated at 24±1° C in the dark in the isolation medium containing 4.5% (w/v) Cellulase 'onozuka' R-10, except when stated otherwise. Protoplasts were released after 16 h by gentle shaking, strained through a double layer of muslin, sedimented by centrifuging at 50 *g* for 1 min and washed three times by centrifuging through isolation medium without Cellulase 'onozuka' R-10. Washed protoplasts were resuspended in a culture medium, comprising the isolation medium without Cellulase 'onozuka' R-10 and either with or without GA_3, at a final density of $1.0 \pm 0.1 \cdot 10^5$ protoplasts ml^{-1} and incubated at 24±1° C in the dark.

Percentages of live protoplasts were estimated by vital staining with 0.01% (w/v) methylene blue as described by Hooley and McCarthy (1980). When quoted in the text these data are means ± standard errors of 3 or 6 replicate experiments (see legends to Tables and Figures). Yields of live protoplasts and total yields of live and dead protoplasts were estimated using a haemocytometer. The diameters and extent of vacuolation of live protoplasts stained with 0.01% (w/v) methylene blue were estimated under Nomarski differential interference contrast optics, and 300 to 500 protoplasts scored in each assay.

Calcofluor White M 2 RS New (American Cyanamid Co., Bound Brook, New Jersey, USA) staining of cellulosic wall material was performed as described by Nagata and Takebe (1970).

Efficiency of protoplast isolation. Aleurone layers were incubated in 6.0% (w/v) CrO_3 at 30±1° C for 16 h with gentle shaking, then dispersed by passing through a Pasteur pipette. After dilution the total number of aleurone cells was estimated using a haemocytometer. The isolation efficiency is expressed as the percentage of total aleurone cells recovered as protoplasts.

Extraction of amylase and RNase. Protoplasts were harvested by centrifuging at 50 *g* for 1 min. The supernatant was immediately cooled on ice, clarified by centrifuging at 3,000 *g* for 15 min, dialysed exhaustively at 4±1° C against 0.1 M sodium acetate buffer pH 5.0 containing 20 mM $CaCl_2$ ('acetate buffer') and stored at −30° C. The pellet of protoplasts was washed once by centrifuging through fresh culture medium, resuspended in ice cold 'acetate buffer' at $1.0 \pm 0.1 \cdot 10^5$ protoplasts ml^{-1} and disrupted by passing 8 times through a 23 G hypodermic needle. After clarifying, by centrifuging at 3,000 *g* for 15 min, the extract was dialysed exhaustively against 'acetate buffer' at 4±1° C and stored at −30° C. Immediately before assaying, the frozen extracts were thawed and clarified by centrifugation at 3,000 *g* for 15 min at 4±1° C.

Amylase assay. Amylase was estimated by incubating 0.1 ml of either an appropriate dilution of clarified extract or, for the blank, 'acetate buffer', with 0.1 ml of 0.2% (w/v) soluble starch in 'acetate buffer' at 30° C for 10 min to 60 min. The reaction was terminated during the linear phase by adding 0.2 ml of 0.5%:0.05% (w/v) $KI:I_2$, the solution diluted to 4 ml with 'acetate buffer' and its A 620 nm recorded at 25° C. Amylase activity is expressed as the reduction in A 620 nm after 10 min incubation at 30° C by extracts prepared from 10^4 live protoplasts (extracted amylase), the culture medium bathing them (secreted amylase), or the sum of the two (total amylase).

RNase assay. RNase was estimated by incubating 0.1 ml of either an appropriate dilution of clarified extract or, for the blank, 'acetate buffer', with 0.4 ml of 0.8 mg ml^{-1} purified yeast RNA (Calbiochem-Behring, La Jolla, USA) in 'acetate buffer' at 37° C for 10 min to 45 min. The reaction was terminated during the linear phase by adding 0.5 ml of 5.0% (w/v) aqueous trichloroacetic acid with 0.6% (w/v) $La(NO_3)_3$. After incubation at 4±1° C for 16 h insoluble material was sedimented by centrifugation at 1,000 *g* for 10 min, the supernatant diluted to 2.5 ml with 'acetate buffer' and its A 260 nm recorded at 25° C. RNase activity is expressed as the increase in A 260 nm after 30 min incubation at 37° C by extracts prepared from 10^4 live protoplasts (extracted RNase), the culture medium bathing them (secreted RNase), or the sum of the two (total RNase).

Amylase and RNase activities in each sample were estimated in three replicate assays and the means calculated. Where standard error is quoted or illustrated in the text this refers to means estimated as described above in replicate experiments.

Osmolality of media. The protoplast isolation and culture media described have a calculated osmolality of approximately 639 mosmol kg^{-1}. Media containing 0.3 M, 0.5 M, 0.6 M and 0.7 M mannitol are approximately 532 mosmol kg^{-1}, 755 mosmol kg^{-1}, 875 mosmol kg^{-1} and 990 mosmol kg^{-1} respectively.

Results

Yields of live protoplasts, their survival and responsiveness to GA_3 were influenced considerably by the composition of the imbibition, isolation and culture media.

Isolation of protoplasts

Enzymes. Freshly prepared aleurone layers were treated with various enzyme preparations. Pectinases such as Macerozyme R-10 (Kinki Yakult Mfg. Co., Nishinomiya, Japan) and Macerase (Calbiochem-Behring, La Jolla, USA), in conjunction with the stabilizer potassium dextran sulphate, failed to dissociate aleurone layers into separated cells. However, Dreiselase (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan), a mixture of pectinases and cellulases, Cellulysin and Cellulysin-B (Calbiochem-Behring, La Jolla, USA), rich in cellulases and hemicellulases, and Micelase (Meiji Seika Kaisha Ltd., Tokyo, Japan) and Cellulase 'onozuka' R-10, both of which possess cellulase, β-1, 3-glucanase, xylanase and other enzyme activities, released cells and protoplasts from the aleurone layers. Yields were reduced when either of the pectinases was used simultaneously with any of the cellulases and were highest using Cellulase 'onozuka' R-10 alone.

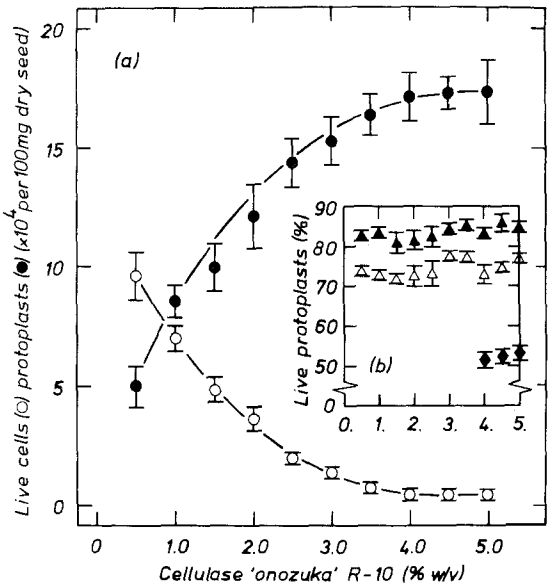

Fig. 1a and b. Yields of live cells and protoplasts as influenced by the concentration of Cellulase 'onozuka' R-10, and survival of the protoplasts during subsequent incubation in vitro. **a** Yields of live cells (○) and protoplasts (●), **b** percentage of protoplasts alive immediately after isolation (▲), after 5 d (△) and, for protoplasts isolated at optimum concentrations of the enzyme, after 12 d (◆). Key symbols and bars denote respectively the means and standard errors of three replicate experiments

Concentration of Cellulase 'onozuka' R-10. When other parameters were optimal, freshly prepared aleurone layers were almost completely dissociated after 16 h incubation with 0.5% (w/v) Cellulase 'onozuka' R-10 yielding large numbers of separated cells and approximately half as many isolated protoplasts (Fig. 1a). At higher concentrations progressively fewer separated cells were recovered while yields of protoplasts increased and were highest between 4.0% to 5.0% (w/v), at these concentrations only very few (<2.0%) of the cells released had not been converted into protoplasts. The percentage of protoplasts that were alive immediately after isolation and their survival during subsequent incubation in vitro was not significantly different over the range of concentrations of Cellulase 'onozuka' R-10 (Fig. 1b). More than 80.0% were alive immediately after isolation and after 5 d only a small proportion of these had died. By 12 d approximately 52.0% of the protoplasts isolated using 4.0% to 5.0% (w/v) of the enzyme were still alive (Fig. 1b).

Absence of cell wall. No cell wall was detected when freshly isolated protoplasts, plasmolysed in the culture medium containing 0.8 M mannitol, were stained with Calcofluor White or examined under Nomarski differential interference contrast optics (Fig. 2a and b). Their osmotic instability was confirmed by rapid

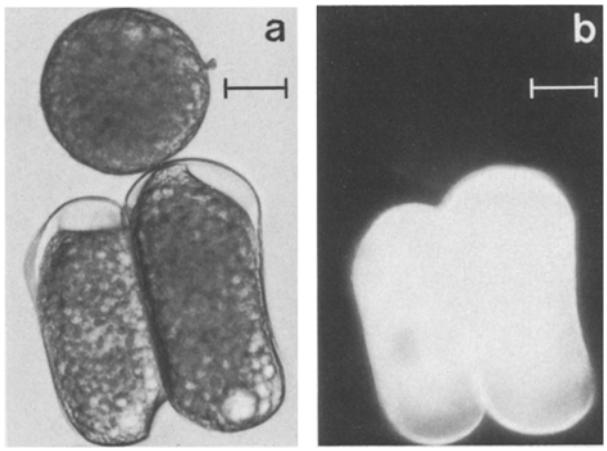

Fig. 2a and b. Light micrographs showing two separated cells and a protoplast obtained after treating aleurone layers with 1.0% (w/v) Cellulase 'onozuka' R-10. After staining with Calcofluor White M 2 RS New the cells and protoplast were plasmolysed in culture medium containing 0.8 M mannitol and observed under visible light using Nomarski optics **a** and ultra-violet light **b**. Bars represent 20 μm

lysis in the mannitol-free culture medium. Cell wall regeneration did not take place during incubation in vitro for up to 5 d under the conditions described here.

Efficiency of protoplast isolation. Under optimum conditions 91.3% to 94.1% of the aleurone cells as determined by CrO_3 digestion, were consistently recovered as protoplast; a yield of approximately $2.9 \cdot 10^6$ aleurone protoplasts from 100 seeds. Using the procedure described protoplasts were also isolated from aleurone layers of *A. sativa* L. cv. 'Pennal' and *Hordeum vulgare* L. cv. 'Maris Otter' (Fig. 6b and c), although 'half seeds' of the latter required 48 h imbibition before aleurones could be recovered.

Effects of mannitol concentration on the yields and survival of protoplasts

Highest numbers of live protoplasts were obtained when mannitol was present in the isolation medium at 0.35 M to 0.50 M i.e. 585 mosmol kg^{-1} to 755 mosmol kg^{-1}, the live protoplasts representing then approximately 78.0% of those recovered (Fig. 3a and b). Yields were reduced in media containing 0.30 M mannitol and fell by up to 45% at above 0.50 M. This reduction could be attributed to a progressive decline in both the proportion of protoplasts that were alive immediately after isolation (Fig. 3b) and the total yields of protoplasts, the latter being associated with incomplete digestion of the tissue. Furthermore, the effect was not overcome by pre-plasmo-

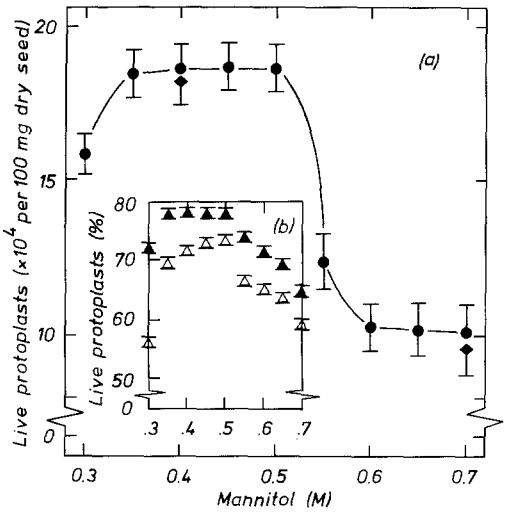

Fig. 3a and b. Yields of protoplasts and their survival during incubation in vitro as influenced by the concentration of mannitol in the isolation and culture media. **a** Yields of live protoplasts obtained without preplasmolysis (●) and after 2 h pre-plasmolysis of 'half seeds' followed by 2 h pre-plasmolysis of aleurones in culture medium containing either 0.4 M or 0.7 M mannitol (◆). **b** Percentage of protoplasts alive immediately after isolation (▲) and after 5 d (△) in culture medium containing the same concentration of mannitol employed during isolation. Of the protoplasts recovered from 'half seeds' pre-plasmolysed in medium containing 0.4 M and 0.7 M mannitol 83.2% and 65.4% respectively were alive immediately after isolation. Key symbols and bars denote respectively the means and standard errors of three replicate experiments

lysing 'half seeds' for 2 h in the culture medium containing 0.70 M mannitol prior to the enzymic removal of endosperm, then subsequently incubating aleurone layers for 2 h in the same medium before isolating protoplasts (Fig. 3a).

Once isolated, all protoplasts survived equally well for 5 d at the different concentrations of mannitol with the exception of those isolated and incubated in media containing 0.30 M mannitol. However, high percentages of live protoplasts were associated only with mannitol concentrations between 0.35 M and 0.50 M (Fig. 3b). More than 73.0% live protoplasts after 5 d could be obtained in culture media with 0.35 M to 0.70 M mannitol, provided that the protoplasts were isolated in a medium containing 0.40 M mannitol and then transferred to the more hypertonic or hypotonic conditions (data not presented).

Effects of L-arginine and $CaCl_2$ on the yields and survival of protoplasts

When 'half seeds' were imbibed in water alone, the aleurone layers prepared and protoplasts isolated and incubated using medium without L-arginine and with only 1.0 mM $CaCl_2$, yields of protoplasts were not optimal (Table 1, see Fig. 1a and b, Fig. 3a and b, and Table 2). Only 58.6 ± 1.6% were alive immediately after isolation and many more had died after 5 d. Yields of protoplasts were no better when 10 mM L-arginine was included in the isolation medium, but their survival was improved when 10 mM L-arginine was included in the culture medium and more so when it was present in both the isolation and culture media. Nevertheless, at best, only 53.1 ± 3.2% of the protoplasts were alive after 5 d (Table 1).

Imbibing 'half seeds' in 50 mM L-arginine improved the yields of live protoplasts, a larger proportion withstanding the rigours of isolation. The survival of these protoplasts was also dependent upon the

Table 1. Effects of L-arginine on the yields and survival of, and amylase production by, aleurone protoplasts. 'Half seeds' were imbibed in water or 50 mM L-arginine, aleurones prepared from them and protoplasts isolated using 3.0% (w/v) Cellulase 'onozuka' R-10 and incubated in media containing 1 mM $CaCl_2$ and either with or without L-arginine. The survival of protoplasts in culture medium containing 10^{-8} M GA_3 was not significantly different from the controls without GA_3, these data have accordingly been combined

Imbibition	Isolation	Live protoplasts[a] 0 h		Culture	Live protoplasts[a] 5 d	10^{-8} M GA_3 5 d Total amylase[a,b]
L-arginine (mM)	L-arginine (mM)	($\times 10^4$ per 100 mg 'half seed')	(%)	L-arginine (mM)	(%)	(units)
–	–	7.9 ± 0.5	58.6 ± 1.6	–	35.6 ± 1.9	0.11 ± 0.05
				10	40.0 ± 1.7	0.23 ± 0.04
–	10	8.1 ± 0.5	59.4 ± 1.8	–	32.4 ± 0.7	0.14 ± 0.08
				10	53.1 ± 0.7	0.33 ± 0.07
50	–	9.6 ± 0.5	71.0 ± 1.4	–	46.3 ± 2.9	0.30 ± 0.09
				10	60.1 ± 1.8	0.84 ± 0.11
50	10	10.4 ± 0.5	72.9 ± 0.9	–	44.4 ± 2.9	0.31 ± 0.08
				10	64.7 ± 1.0	0.97 ± 0.10

[a] Mean ± standard error of 6 replicate experiments
[b] Minus control values

Table 2. Effects of L-arginine and $CaCl_2$ on the yields and survival of, and amylase production by, aleurone protoplasts. 'Half seeds' were imbibed in 50 mM L-arginine (except[c]), aleurone layers prepared from them and protoplasts isolated using 3.0% (w/v) Cellulase 'onozuka' R-10 and incubated using media with and without added L-arginine and $CaCl_2$. The survival of protoplasts in culture medium containing 10^{-8} M GA_3 was not significantly different from the controls without GA_3, these data have accordingly been combined

Isolation and culture media		Live protoplasts[a]	Live protoplasts[a]		10^{-8} M GA_3 Total amylase[a,b]
L-arginine (mM)	$CaCl_2$ (mM)	($\times 10^4$ per 100 mg 'half seed')	0 h (%)	5 d (%)	5 d (units)
–	20	12.7±0.7	73.2±1.6	66.3±2.6	1.68±0.12
–	50	11.7±0.3	73.1±1.3	66.7±3.0	1.70±0.10
10	20	14.6±1.0	82.8±0.7	77.2±1.1	2.23±0.10
10	50	13.8±0.6	81.3±1.0	78.1±4.2	2.01±0.10
50	1	14.4±0.7	84.0±1.0	78.2±1.8	1.17±0.21
10[c]	20	12.4±0.2	69.8±2.7	66.7±1.9	1.81±0.10

[a] Mean±standard error of 6 replicate experiments
[b] Minus control values
[c] 'Half seeds' were imbibed in 50 mM $CaCl_2$

presence of 10 mM L-arginine in the culture medium (Table 1).

Total yields of protoplasts, their stability during isolation and survival during incubation, were improved further when the isolation and culture media were modified by increasing $CaCl_2$ from 1.0 mM (Gamborg et al. 1968) to 20 mM or 50 mM, and were optimal when either (i) 10 mM L-arginine was also included with the higher concentrations of $CaCl_2$, or (ii) 50 mM L-arginine was present with 1.0 mM $CaCl_2$ (Table 2).

Interestingly, yields of live protoplasts from 'half seeds' imbibed in 50 mM $CaCl_2$ were almost as high as yields from 'half seeds' imbibed in 50 mM L-arginine (Table 2).

The effects of L-arginine and $CaCl_2$ were not exclusive to the modified Gamborg's B-5 medium. Yields and survival of protoplasts were improved considerably when 10 mM L-arginine and 20 mM $CaCl_2$ were included in a simplified medium consisting of 0.40 M mannitol and 2.0% (w/v) glucose with and without 4.5% (w/v) Cellulase 'onozuka' R-10 (data not presented).

GA_3-induced production and secretion of enzymes

Survival of protoplasts, $CaCl_2$ and total amylase. Control protoplasts without GA_3 gave only very low levels, 0.01 to 0.07 units, of total amylase after 5 d. When 10^{-8} M GA_3 was included in the culture media protoplasts produced and secreted amylase. Responsiveness to GA_3 however was correlated with treatments that improved the yields of live protoplasts and their survival during incubation (Tables 1 and 2). Furthermore, 20 mM or 50 mM $CaCl_2$ in the isolation and culture media stimulated the total amylase from GA_3-treated protoplasts over and above its effects on protoplast yields and survival (Table 2).

Time course of amylase and RNase production and secretion. Protoplasts incubated in the culture medium containing $1.61 \cdot 10^{-7}$ M GA_3 did not produce or secrete significant amounts of amylase until the third day, or RNase until the fourth day; thereafter, the enzymes increased simultaneously in both the protoplasts and the culture medium. Control protoplasts produced and secreted only very small amounts of amylase and RNase over the 5 d period (Fig. 4a and b).

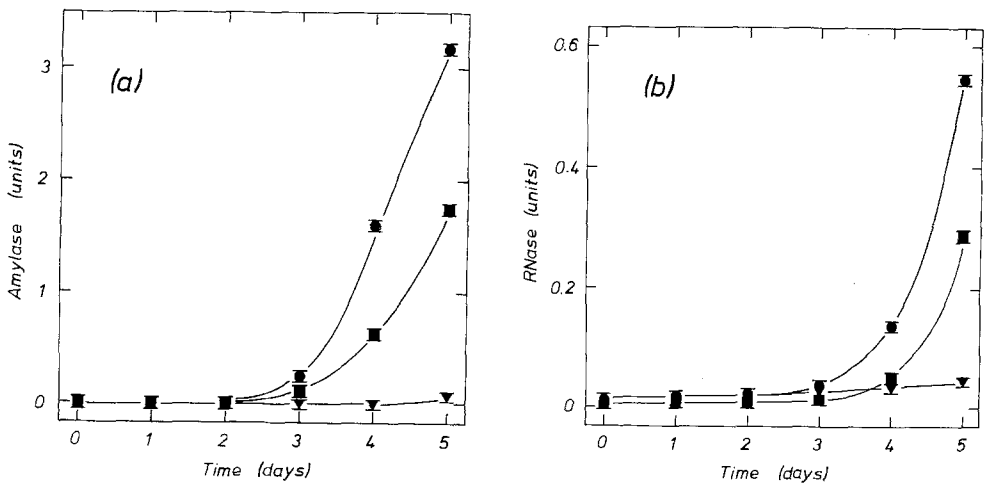

Fig. 4a and b. Time course of amylase **a** and RNase **b** production and secretion. Total (●) and secreted (■) enzymes from protoplasts incubated in culture medium containing $1.61 \cdot 10^{-7}$ M GA_3. Total enzymes (▼) from protoplasts incubated without GA_3. Key symbols and bars denote respectively the means and standard errors of three replicate experiments

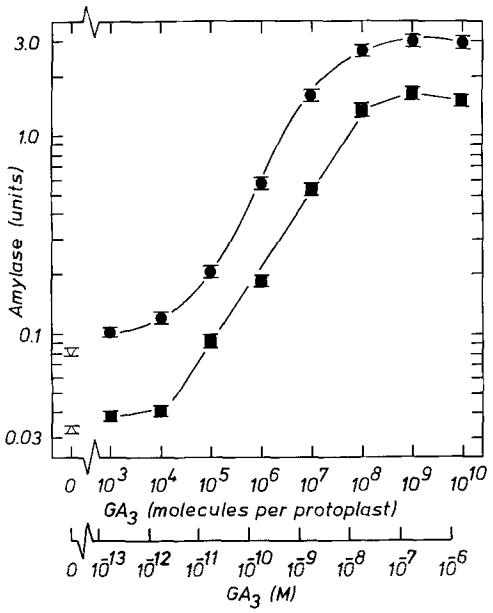

Fig. 5. Dose response. Total (●) and secreted (■) amylase from protoplasts incubated for 5 d in culture medium with a range of concentrations of GA_3 and from controls (▽△) without GA_3. The amount of the hormone is presented as both the molar concentration, and the calculated number of molecules available to each protoplast. Key symbols and bars denote respectively the means and standard errors of three replicate experiments

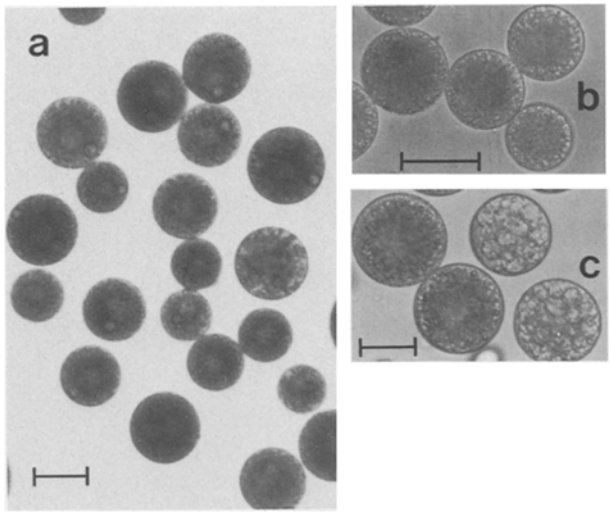

Fig. 6a, b and c. Light micrographs taken, using Nomarski optics, of freshly isolated aleurone protoplasts of **a** *A. fatua*, **b** *A. sativa* L. cv. 'Pennal' and **c** *H. vulgare* L. cv. 'Maris Otter'. Bars represent 50 μm (**a** and **b**) and 25 μm (**c**)

Dose response. Amylase production and secretion was significantly higher than the controls when $1.61 \cdot 10^{-13}$ M GA_3 was included in the culture medium, a concentration corresponding to approximately 1,000 molecules of GA_3 available to each protoplast (Fig. 5). With increasing concentrations of GA_3, amylase production increased and a progressively larger proportion of the total amylase was secreted into the culture medium. Amylase production and secretion reached a maximum at approximately $1.61 \cdot 10^{-7}$ M GA_3 (Fig. 5).

Vacuolation of protoplasts

Freshly isolated protoplasts were densely cytoplasmic with abundant organelles and no discernible vacuolation (Fig. 6a, b and c). During incubation in the culture medium containing 0.4 M mannitol protoplasts underwent vacuolation (Fig. 7a, b, c, d and e) and on the basis of their appearance were assigned to one of four groups: (a) non-vacuolate, (b) densely cytoplasmic with numerous aleurone grain-vacuoles, (c) vacuolate, protoplasts containing between 5 to 15 vacuoles/aleurone grain-vacuoles, and (d) highly vacuolate, protoplasts with a large central vacuole and as many as 4 smaller aleurone grain-vacuoles (Fig. 8). Vacuolation did not occur synchronously in all the protoplasts. However, as the proportion of non-vacuolate protoplasts (group a) declined the proportion of group b and group c protoplasts increased accordingly. Highly vacuolate protoplasts (group d) appeared on the second day and by 5 d the population consisted of vacuolate (group c), and highly vacuolate (group d) protoplasts. Vacuolation was markedly stimulated by $1.61 \cdot 10^{-7}$ M GA_3 (Fig. 7a, b, c, d and e, and Fig. 8).

Protoplasts also increased in size, reaching a maximum at 3 d when they were approximately 50% larger in diameter than immediately after isolation. Protoplast diameter was at no time correlated with the extent of vacuolation (Table 3, Fig. 7a, b, c, d and e, and Fig. 8).

The influence of mannitol concentration on the production and secretion of amylase and RNase, and on vacuolation

Protoplasts incubated without GA_3 exhibited only very low levels of amylase and RNase when mannitol was present at 0.35 M to 0.70 M (Fig. 9b, Fig. 10b). Total amylase and RNase from GA_3-treated protoplasts were highest in culture media containing 0.35 M mannitol, declined with increasing concentrations of mannitol and were not significantly higher than controls without GA_3 at 0.60 M to 0.70 M. This inhibition was not overcome by increasing the concentration of GA_3 100 fold in the plateau region of the dose response curve (Fig. 9b, Fig. 10b, see Fig. 5).

Secretion of the enzymes was inhibited more markedly than their production. With increasing concentrations of mannitol progressively less of the total

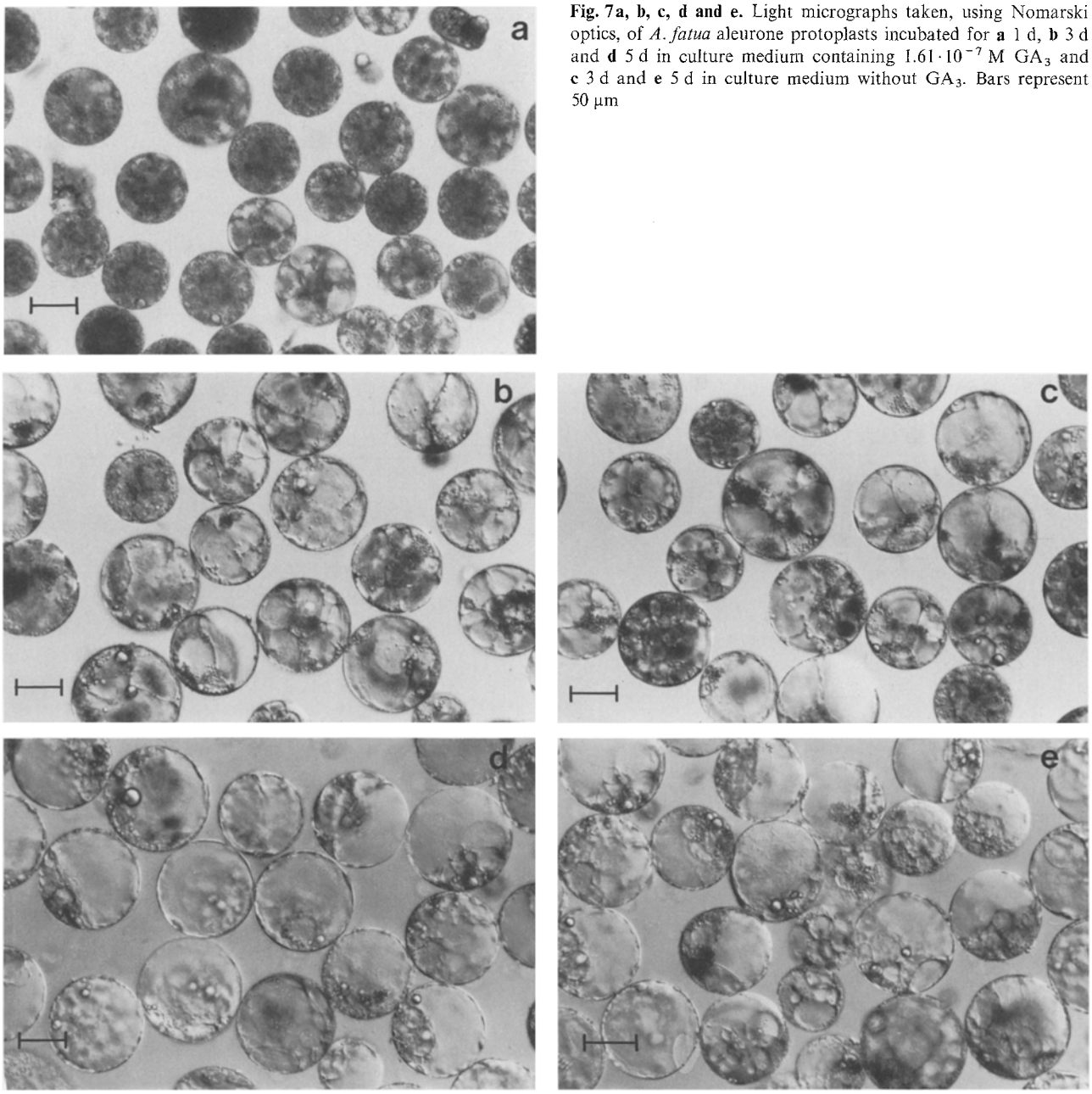

Fig. 7a, b, c, d and e. Light micrographs taken, using Nomarski optics, of *A. fatua* aleurone protoplasts incubated for **a** 1 d, **b** 3 d and **d** 5 d in culture medium containing $1.61 \cdot 10^{-7}$ M GA_3 and **c** 3 d and **e** 5 d in culture medium without GA_3. Bars represent 50 μm

amounts of enzymes produced were secreted, a larger proportion remaining accordingly within the protoplasts (Fig. 9a and b, Fig. 10a and b).

Vacuolation of protoplasts was also inhibited by increasing concentrations of mannitol (Fig. 11). In fact, in media containing 0.70 M mannitol, less than 50% of the protoplasts had developed aleurone grain-vacuoles (group b) after 5 d; the remainder were non-vacuolate (group a) (Fig. 11, Fig. 12). Protoplasts treated with $1.61 \cdot 10^{-8}$ M GA_3 were more highly vacuolate than the controls at all concentrations of mannitol except 0.70 M (Fig. 11).

Discussion

Perhaps it is not surprising that highest yields of live aleurone protoplasts can be obtained only over a fairly narrow range of concentrations of osmoticum in the isolation medium, even though, once isolated, protoplasts will tolerate a wider range of osmotic pressures (Fig. 3a and b). In fact, optimum osmolalities have been demonstrated for the yields of mesophyll protoplasts from several plant species (Shepard and Totten 1975; Hooley 1978; Mühlbach et al. 1977; Hampp and Ziegler 1980), and indeed may vary with

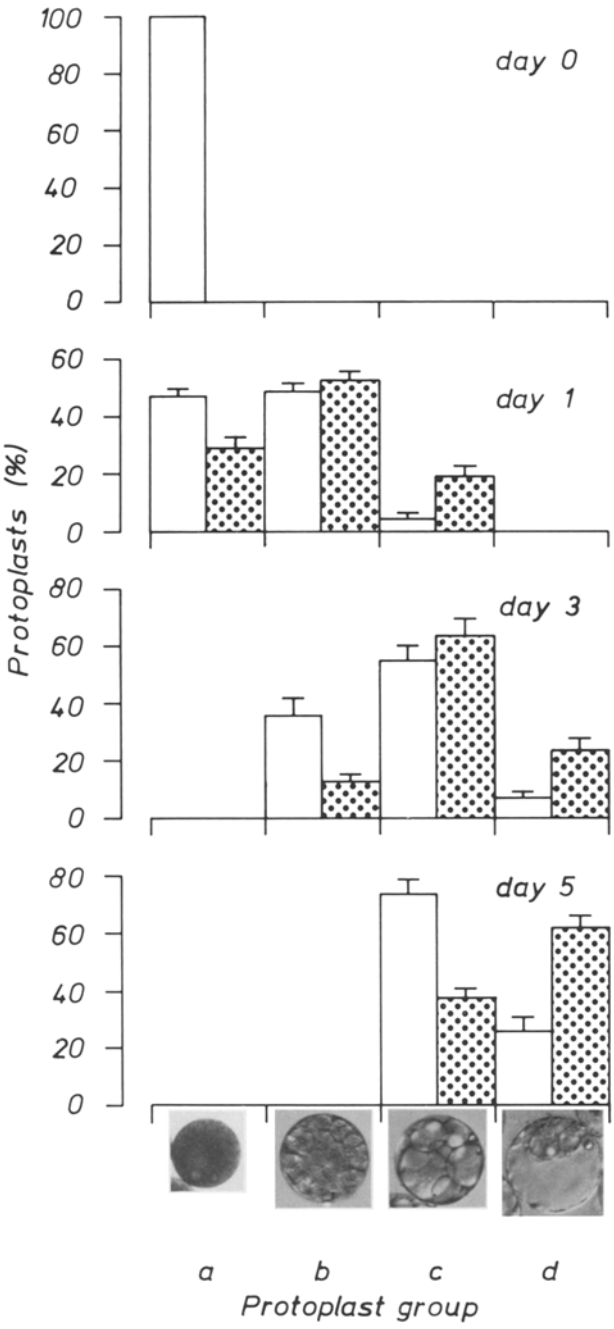

Fig. 8. Vacuolation of protoplasts during incubation in vitro. Protoplasts were examined and assigned to one of the four groups a, b, c or d, immediately after isolation and after 1 d, 3 d and 5 d in culture medium without GA_3 (unshaded histograms) and with $1.61 \cdot 10^{-7}$ M GA3 (shaded histograms). Bars denote standard errors of three replicate experiments

the conditions under which the plant material has been grown (Hooley 1978), the concentration of the enzymes employed (Kirby and Cheng 1979) and the state of differentiation of the tissue (Senn and Pilet 1980).

Table 3. Increase in diameter of protoplasts during incubation in vitro. The diameters of protoplasts of each of the four groups and at each sampling time were distributed normally about the mean

Time (d)	Protoplast group[a]	Diameter[b]	
		without GA_3 (μm)	$1.61 \cdot 10^{-7}$ M GA_3 (μm)
0	a	64.4±4.5	
1	a	66.8±6.2	72.2±7.3
	b	70.1±5.3	76.6±5.5
	c	73.8±6.1	76.9±5.3
3	b	93.7±7.9	98.0±7.7
	c	90.2±7.9	96.8±6.5
	d	88.8±5.9	92.7±7.2
5	c	88.4±6.4	98.4±8.0
	d	84.5±5.5	91.4±7.4

[a] See Fig. 8
[b] Mean ± standard error of three replicate experiments

Because pre-plasmolysis does not overcome the reduction in yields of *A. fatua* aleurone protoplasts at the high osmolalities (Fig. 3a) it seems unlikely that these protoplasts are killed by phytotoxic levels of Cellulase 'onozuka' R-10 (Cocking and Evans 1973) arising through stimulated plasmalemma infolding and vesicle accumulation (Withers and Cocking 1972). It is perhaps more likely that aleurone protoplasts sustain irreversible damage at the plasmalemma as plasmodesmata or regions of attachment to the cell wall are rapidly severed during plasmolysis in high concentrations of osmoticum (Zeiger and Hepler 1976; Colman and Mawson 1978, also see Fig. 2a and b).

Before protoplasts can be isolated from aleurone layers of mature dry grain of *A. fatua* the 'half seeds' must first be imbibed. The inclusion of 50 mM L-arginine or 50 mM $CaCl_2$ in the imbibition medium enables a larger proportion of the protoplasts to survive during isolation (Table 1). Similar effects of L-arginine, $CaCl_2$ and other pre-treatments on yields of protoplasts from various plant tissues have been reported (Kaur-Ṣawhney et al. 1977; Wallin et al. 1977; Fukunaga and King 1978; Schmidt and Poole 1980; Senn and Pilet 1980) and attributed to senescence retardation, modifications in cell wall metabolism that favour protoplast isolation and the prevention of inhibition of protoplast-releasing enzymes by phenolics or their oxidised derivatives.

During the isolation and incubation of *A. fatua* aleurone protoplasts a combination of 10 mM L-arginine and 20 mM $CaCl_2$ ensures both optimum yields and long-term survival of the protoplasts (Table 1 and 2). It is now well documented that L-arginine,

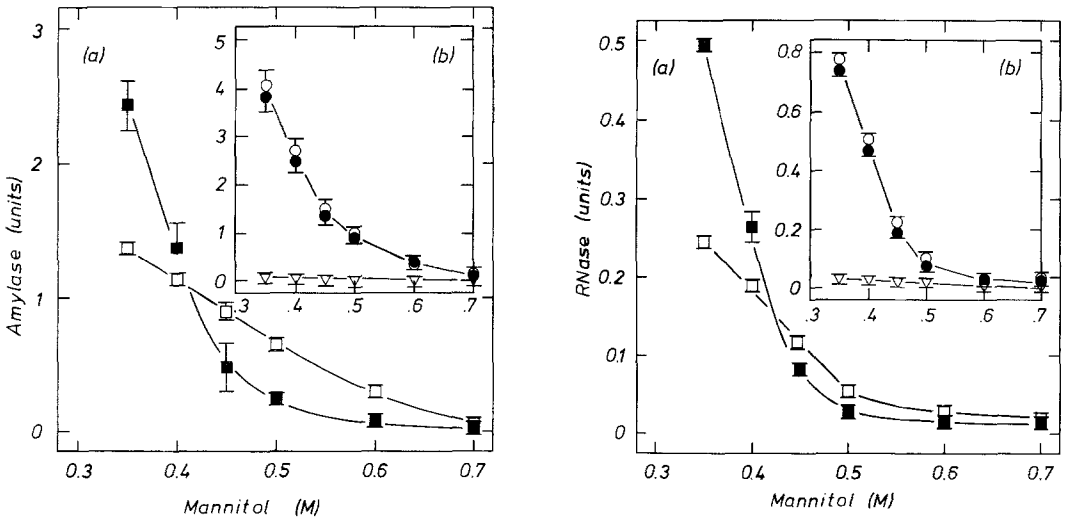

Fig. 9a and b, Fig. 10a and b. The effect of mannitol concentration in the culture medium on the production and secretion of amylase (Fig. 9a and b) and RNase (Fig. 10a and b). Protoplasts were isolated in medium containing 0.40 M mannitol and transferred to culture media containing a range of concentrations of mannitol both with and without GA_3. **a** Extracted enzymes (□), secreted enzymes (■), after 5 d incubation in mediam containing $1.61 \cdot 10^{-8}$ M GA_3. **b** Total enzymes after 5 d incubation in media without GA_3 (▽), with $1.61 \cdot 10^{-8}$ M GA_3 (●) and $1.61 \cdot 10^{-6}$ M GA_3 (○). Key symbols and bars denote respectively the means and standard errors of three replicate experiments

other dibasic amino acids and polyamines, delay degenerative changes in *A. sativa* L. cv. Victory mesophyll protoplasts (Fuchs and Galston 1976; Galston et al. 1978), either by ionic stabilization of the plasma membrane (Kaur-Sawhney et al. 1977; Altman et al. 1977), prevention of the increase in RNase following protoplast isolation (Kaur-Sawhney et al. 1977, 1978; Galston et al. 1978), altered nucleic acid metabolism (Altman et al. 1977; Kaur-Sawhney et al. 1977; Galston et al. 1978) or an interaction with ribosomes (Kaur-Sawhney et al. 1977).

The stabilization of *A. fatua* aleurone protoplasts by $CaCl_2$ is consistent with its reported effects on other plant protoplasts (Ruesink 1971; Constabel 1975; Evans and Cocking 1977; von Arnold and Erikson 1977; Rose 1980) and might be mediated through surface charge neutralization (Nagata and Melchers 1978) or altered membrane fluidity (Boss and Mott 1980). Furthermore, the importance of $CaCl_2$ in sustaining optimum GA_3-induced production and secretion of amylase by the aleurone protoplasts is in accordance with the similar requirement for $CaCl_2$ by aleurone layers of *H. vulgare* L. cv. Himalaya (Chrispeels and Varner 1967).

Aleurone layers of dormant *A. fatua* seed respond to treatment with GA_3 by producing and secreting α-amylase after a lag period of approximately 48 h to 55 h (Naylor 1966; Chen and Chang 1972). In this respect the behaviour of protoplasts isolated from *A. fatua* aleurones is remarkably similar (Fig. 4a). Aleurone layers of *H. vulgare* are known to secrete several hydrolytic enzymes, including RNase, in response to GA_3 (see the reviews of Jones 1973; Bewley and Black 1978). It is also known that high levels of RNase are induced by osmotic stress in mesophyll protoplasts of *N. tabacum* L. cv. Xanthi (Lázár et al. 1973; Premecz et al. 1977) and as a result of post-isolational senescence in *A. sativa* mesophyll protoplasts (Kaur-Sawhney et al. 1977; Altman et al. 1977; Galston et al. 1978), although cycloheximide, kinetin, L-arginine and polyamines will prevent the increases in RNase in these protoplasts.

Aleurone protoplasts of *A. fatua*, isolated and incubated under the conditions described here, exhibit only very low levels of RNase in the absence of GA_3. When treated with the hormone they produce and secrete RNase after a lag period of 3 d to 4 d (Fig. 4b). Production and secretion do not appear to be separated temporally as they are in *H. vulgare* aleurones (Chrispeels and Varner 1967), and it is not known yet whether this reflects differences between aleurone layers of *A. fatua* and *H. vulgare* or is a feature of isolated aleurone protoplasts.

The dose response relationship between GA_3 and amylase production and secretion by *A. fatua* aleurone protoplasts (Fig. 5) is comparable with that of *H. vulgare* aleurone layers (Chrispeels and Varner 1967; Jacobsen and Varner 1967; Mapelli and Ranieri 1978). However, isolated protoplasts respond to considerably lower concentrations of the hormone.

When aleurone layers of *H. vulgare* are treated with GA_3, aleurone grains lose their typical spherical

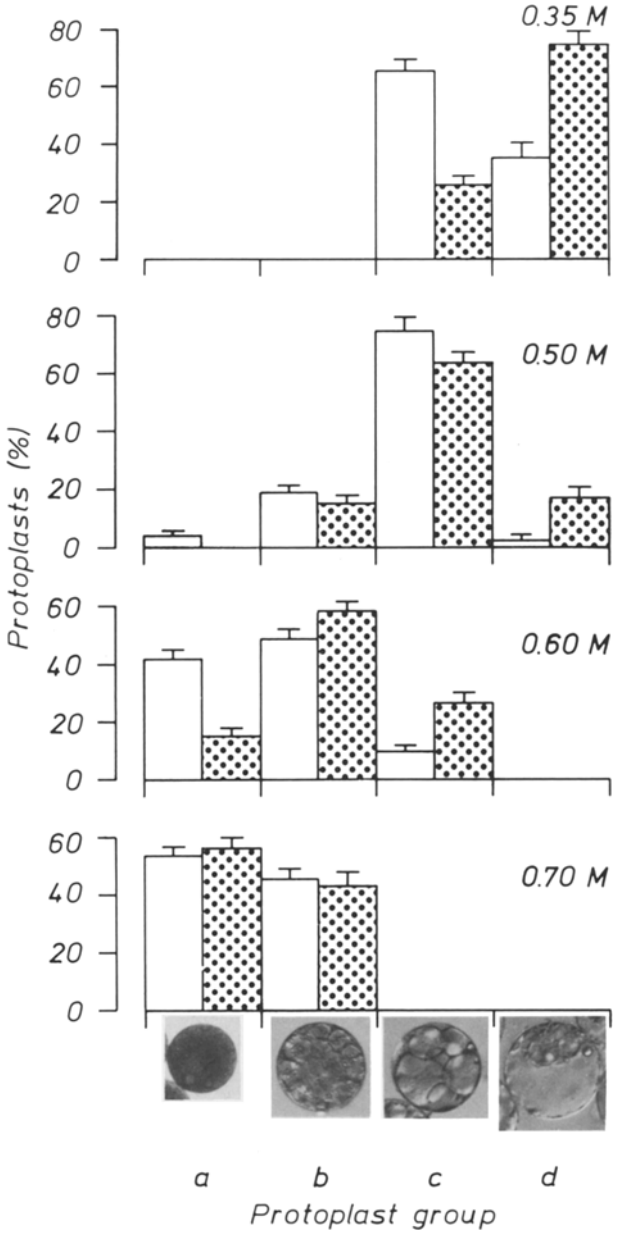

Fig. 11. The influence of mannitol concentration in the culture medium on vacuolation of protoplasts. Protoplasts were isolated in a medium containing 0.40 M mannitol and transferred to culture media containing a range of concentrations of mannitol. After 5 d protoplasts incubated without GA_3 (unshaded histograms) and with $1.61 \cdot 10^{-8}$ M GA_3 (shaded histograms) were examined and assigned to one of the four groups a, b, c, or d. Bars denote standard errors of three replicate experiments

appearance within 1 h to 2 h and increase in volume as stored proteins are hydrolysed (Jones 1969b). Thereafter, aleurone grain-vacuoles expand and fuse (Jones 1969c) and by 30 h to 36 h each cell possesses a large central vacuole and other small vacuoles in the peripheral cytoplasm (Jones and Price 1970). A similar sequence of events occurs, albeit more slowly,

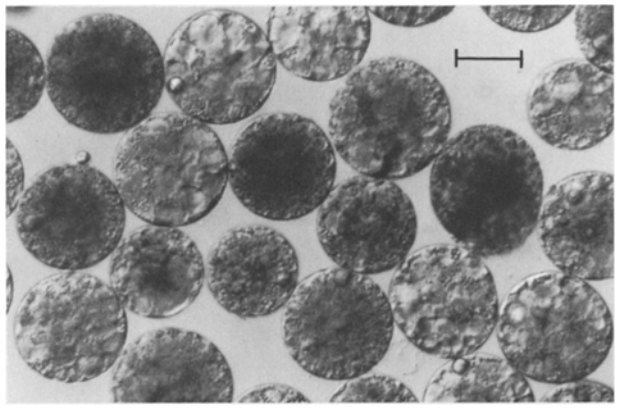

Fig. 12. Light micrograph taken, using Nomarski optics, of protoplasts incubated for 5 d in culture medium containing 0.70 M mannitol and $1.61 \cdot 10^{-8}$ M GA_3. Bar represents 50 µm

during incubation of *A. fatua* aleurone protoplasts in the culture medium without GA_3 and is stimulated by addition of the hormone (Fig. 7a, b, c, d and e, and Fig. 8). It is not yet clear whether vacuolation of the control protoplasts without GA_3 reflects their dedifferentiation (Gigot et al. 1975), or occurs in the intact *A. fatua* aleurone layer.

Polyethylene glycol, mannitol, glucose and maltose inhibit GA_3-induced α-amylase production by aleurone layers of *H. vulgare* (Jones 1969a; Jones and Armstrong 1971), reportedly either by inhibiting the hydrolysis of aleurone grain reserves and hence reducing the availability of substrates for de novo protein synthesis (Jones 1969a), or by impairing mRNA translational capacity through dissociation of membrane-bound polyribosomes (Jones et al. 1973; Armstrong and Jones 1973). Failure of separated aleurone cells and protoplasts of *H. vulgare* and *A. sativa* to respond to GA_3 has been attributed, in part, to osmotic inhibition by the plasmolyticum in the culture medium (Jones et al. 1973; Eastwood 1977). In fact, it now seems certain that amylase and RNase production by *A. fatua* aleurone protoplasts are indeed regulated osmotically, probably by the same mechanism that operates in the intact tissue (Fig. 9a and b, Fig. 10a and b, Fig. 11 and Fig. 12).

In conclusion, protoplasts isolated from aleurone layers of *A. fatua* offer unique prospects for investigations of the hormonal control of this highly specialized secretory tissue, and represent important new experimental tools for the seed biologist.

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