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INHIBITION OF MEMBRANE DAMAGE DUE TO PHOSPHOLIPASE ACTIVITY DURING FRACTIONATION OF WHEAT ALEURONE TISSUE

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Abstract—Fractionation of [Me-^{14}C]choline-labelled wheat aleurone tissue in the absence of phospholipase inhibitors resulted in a 60% loss of label from phosphatidylcholine and even larger losses of NADH–cytochrome *c* reductase activity from microsomal fractions. Several putative inhibitors of phospholipase D were tested for their ability to protect the membranes during fractionation. The addition of choline and *O*-phosphorylcholine, together with glycerol-1-phosphate to inhibit any phosphatidic acid phosphatase activity, proved to be the best protectants. In tissue from freshly imbibed seeds, however, the addition of *p*-chloromercuribenzoate to a cocktail of inhibitors was necessary for the best recovery of radiolabelled membranes. Effects of the inhibitors on phospholipase D activity in cell free extracts were studied in an attempt to confirm the enzyme as the cause of membrane damage.

INTRODUCTION

The response of cereal aleurone tissue to gibberellins involves changes in cellular ultrastructure and in membrane metabolism [1]. In order to study these processes fully, reliable methods are needed for the sub-cellular fractionation of the tissue. Our first attempts to adopt existing methods from the literature led to unacceptably large losses of some phospholipids and to agglutination of particles in some of the sub-cellular fractions. Destructive activity by endogenous phospholipases upon sub-cellular fractions from plant tissues has been documented [2, 3]. Phospholipase D (PLase D) in particular can be very active in this respect. The presence of high activities of PLase D in wheat aleurone tissue [4] therefore made it a likely cause of our difficulties. A lysophospholipase has also been reported to be present in barley aleurone tissue [5].

Despite the known dangers arising from the activities of phospholipases and the existence of PLase inhibitors [6], it is surprising that many sub-cellular studies have been conducted without appropriate precautions being taken. A notable exception was the study of soybean hypocotyls by Scherer and Morre [7]. They successfully inhibited PLase D activity by adding 4% choline plus 4% ethanolamine to isolation media, and phosphatidic acid phosphatase (PAPase) was inhibited by 10 mM glycerol-1-phosphate (G-1-P). A procedure for the isolation of microsomal membranes from barley aleurone tissue has been described by Jones [8]. An important feature of his method was the use of gel filtration to separate a particulate fraction from water-soluble components of the cell. This procedure confers several advantages over differential centrifugation. Not least of these is the avoidance of centrifugation as a means of removing Ca^{2+} ions from the particulate fraction. This avoidance of pelleting by centrifugation reduces the agglutination of the particulate material. Jones did not, however, use PLase inhibitors in his media.

In the present study we have attempted to redress this situation with respect to wheat aleurone tissue. Using the gel filtration procedure from Jones [8], we have investigated the efficacies of various phospholipase inhibitors for the production of undamaged sub-cellular fractions. The resultant technique has allowed us to study phospholipid synthesis in relation to membrane formation in the aleurone tissue [9].

RESULTS

Damage to membranes during tissue fractionation was monitored following labelling of phosphatidyl choline (PC) with [Me-^{14}C]choline. Aleurone tissue from 48 hr embryoectomized seed was used for the first series of experiments in which lipid was extracted from homogenates immediately following their preparation. When homogenization was carried out in the absence of inhibitors, *ca* 42% of radiolabelling was lost from the lipid fraction compared with the control value obtained by extracting the labelled tissue prior to homogenization (Table 1). Addition of potential PLase inhibitors to the homogenization medium improved the recovery. G-1-P was added in every case as a putative inhibitor of PAPase [7]. Various compounds with molecular characteristics similar to the phosphocholine moiety of PC were also added. (PLase D assumedly binds to the phosphocholine headgroup of its PC substrate and it might therefore be inhibited by the chemical analogues.) Choline plus ethanolamine were tested as a cross-reference to the results of Scherer and Morre [7]. 2-Propanol was tested because secondary alcohols are known inhibitors of PLase D. Each of the tested compounds improved the recovery of labelled phospholipid. Choline plus ethanolamine gave a

Table 1. Recovery of radiolabelled lipid following homogenization of aleurone tissue

Inhibitor	Radio activity (dpm)	Recovery (%)
None	17 830	58
2-Propanol (4%)	22 020	72
25 mM L-threonine + 10 mM G-1-P	22 500	73
Choline (4%) + ethanolamine (4%)	23 470	77
25 mM *O*-phosphoryl-DL-threonine + 10 mM G-1-P	25 780	84
25 mM *O*-phosphorylethanolamine + 10 mM G-1-P	25 840	84
25 mM DL-carnitine + 10 mM G-1-P	25 860	84
25 mM PCHOL + 10 mM G-1-P	28 200	92
Control (tissue directly extracted)	30 670	—

The experiment was carried out on tissue from 50 embryoectomized seeds incubated for 48 hr. The tissue was radiolabelled with 2.5 μCi [Me-^{14}C]choline. Each value is the average from two separate experiments.

recovery of 77% *O*-phosphoryl choline (PCHOL) plus G-1-P was the most effective inhibitor, giving a 92% recovery. A separate experiment (not reported) showed that PCHOL was most effective at concentrations above 20 mM. A concentration of 25 mM was therefore retained in all subsequent experiments.

In another series of experiments, the efficiences of the inhibitors were investigated through a sequence consisting of homogenization, filtration, 800 *g* centrifugation and gel filtration chromatography. This sequence yielded cell debris, 800 *g* pellet and a particulate fraction from the gel filtration. The results from 48 hr embryoectomized seed are shown in Table 2. In the absence of inhibitors, the total recovery of lipid-associated radioactivity in all of the fractions was only 40% of the control value. Use of G-1-P alone produced a small improvement in the value for the particulate fraction, while a mixture of choline and ethanolamine gave a considerable improvement. PCHOL in the presence of G-1-P remained the best inhibitor, however. Using this combination of inhibitors the total recovery of radioactive lipids in all of the fractions was 99%. Two other known inhibitors of PLase D were also tested in these experiments. These were the cationic detergent cetyltrimethylammonium bromide and the basic protein protamine sulphate [10, 11]. In the presence of these inhibitors very little radioactivity was recovered from the particulate fraction, presumably due to dispersion of the particulate material into low M_r products which were not eluted in the void volume from the gel filtration column.

PCHOL and G-1-P were considerably less effective when used in media for the sub-cellular fractionation of aleurone tissue from 14 hr embryoectomized seed. Only 44% of the total radiolabelled lipid was recovered using these inhibitors (Table 3). Homogenization and fractionation in the absence of inhibitors gave a 25% recovery,

Table 2. Recovery of radiolabelled lipid following cell fractionation

Inhibitor	Radioactivity (dpm)			Total recovery (%)
	Cell debris	800 *g* pellet	Particulate fraction	
None	6830	3390	18950	40
10 mM G-1-P	—	—	22560	—
Choline (4%) + ethanolamine (4%) + 10 mM G-1-P	—	—	50000	—
25 mM PCHOL + 10 mM G-1-P	11130	5620	55040	99
2.5 mM cetyltrimethylammonium bromide + 10 mM G-1-P			< 1000	—
Protamine sulphate (0.8 mg/ml) + 10 mM G-1-P			< 1000	—
Control (tissue directly extracted)	←	72240	→	

The experiment was carried out on tissue from 100 embryoectomized seeds incubated for 48 hr. The tissue was radiolabelled with 5 μCi [Me-^{14}C]choline. Each value is the average from two separate experiments.

Table 3. Recovery of radiolabelled lipid following cell fractionation

Inhibitor	Radioactivity (dpm) Cell debris	800 *g* pellet	Particulate fraction	Total recovery (%)
None	4630	3250	20151	25
25 mM tetramethylammonium bromide	—	—	18030	—
10 mM KF	—	—	22820	—
1 mM *p*CMB	—	—	30800	—
10 mM G-1-P + 1 mM *p*CMB	—	—	36200	—
25 mM PCHOL + 10 mM G-1-P	5890	3670	38600	44
25 mM PCHOL + 10 mM G-1-P + 1 mM *p*CMB	—	—	40250	—
Choline (2%)	—	—	42050	—
Choline (2%) + 10 mM G-1-P + 1 mM *p*CMB	8270	4180	57100	63
Control (tissue directly extracted)	←——110000——→			

The experiment was carried out on tissue from 100 embryoectomized seeds incubated for 14 hr. The tissue was radiolabelled with 5 μCi [Me-^{14}C]choline. Each value is the average from two separate experiments.

indicating high levels of PLase activity. General enzyme inhibitors such as KF and *p*-chloromercuribenzoate (*p*CMB), either alone or in combination with PCHOL and G-1-P, did not improve the recovery. In contrast to its rating with the 48 hr tissue, choline was more effective than PCHOL plus G-1-P, for the particulate fraction at least. The best combination of inhibitors was a mixture of choline, G-1-P and *p*CMB. This cocktail gave 63% recovery of radioactivity.

Using aleurone tissue from 48 hr embryoectomized seed the efficacy of PCHOL plus G-1-P was examined during the preparation of particulate fractions for isopycnic centrifugation. A radiolabelled particulate fraction from gel filtration, prepared either in the presence or absence of inhibitors, was used for these experiments. The centrifugation was carried out in medium containing no inhibitors. In the absence of inhibitors a considerable proportion of the radioactivity remained in the sample band (Fig. 1). This was associated with a large band of turbidity which hardly penetrated the gradient. Very little turbidity and little radioactivity equilibrated at buoyant densities corresponding to endoplasmic reticulum (ER) or mitochondria. The overall recovery of radioactivity from the centrifugation was 90%. When inhibitor-containing media were used, most of the radioactivity, associated with a distinct band of turbidity, equilibrated at buoyant density 1.11–1.12 g/cm^3. A smaller peak, also associated with a band of turbidity, equilibrated at 1.17 g/cm^3. Additionally, a large amount of radioactivity was found in the lipid pad which collected at the surface of the centrifuged sample. The overall recovery of radioactivity from this centrifugation was 97%. The distributions of marker enzyme activities are presented in Fig. 2. A peak of NADH–cytochrome *c* reductase, at 1.11–1.12 g/cm^3, identified microsomal membranes and corresponded to the largest peak of radioactivity. A second peak of enzyme activity, present at 1.17 g/cm^3, was associated with mitochondria and corresponded to the smaller peak of radioactivity. Mitochondrial cytochrome oxidase activity also peaked at 1.17 g/cm^3. When the inhibitors were omitted from the media (Fig. 2b), the peaks of NADH–cytochrome *c* reductase at 11.11–1.12 and 1.17 g/cm^3 were greatly reduced and another peak of activity appeared in the sample band. A large overall loss (66%) of enzyme activity resulted from the omission of the inhibitors. The marker enzyme studies thus confirm the radiotracer results in Fig. 1.

Further confirmation that the inhibitors protect ER membranes from damage was obtained by isopycnic centrifugation in high-Mg^{2+} media (Fig. 3). Under this

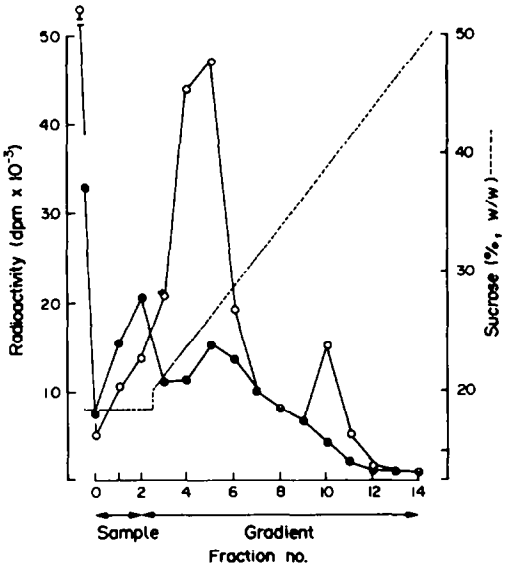

Fig. 1. Distribution of radioactivity following centrifugation on a sucrose gradient. ● Isolation medium containing no inhibitors; ○ isolation medium containing 25 mM PCHOL + 10 mM G-1-P.

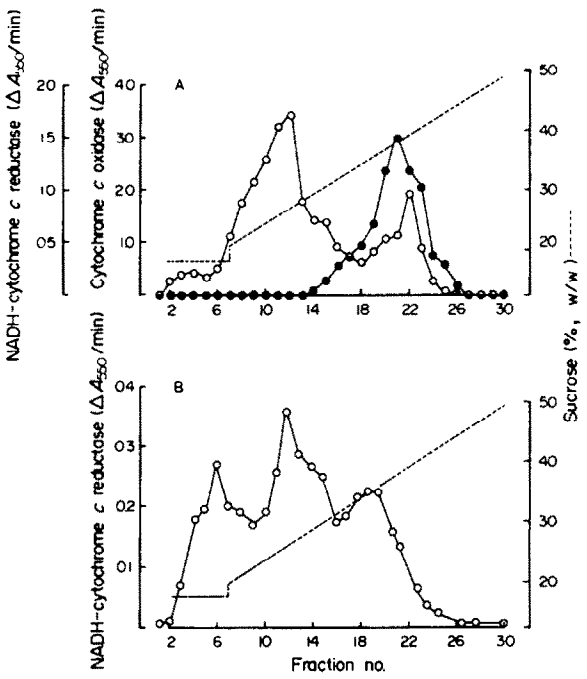

Fig. 2. Distribution of marker enzyme activities following centrifugation on a sucrose gradient. A. Isolation medium containing 25 mM PCHOL + 10 mM G-1-P; B. isolation medium containing no inhibitors. ○ NADH-cytochrome *c* reductase; ● cytochrome oxidase.

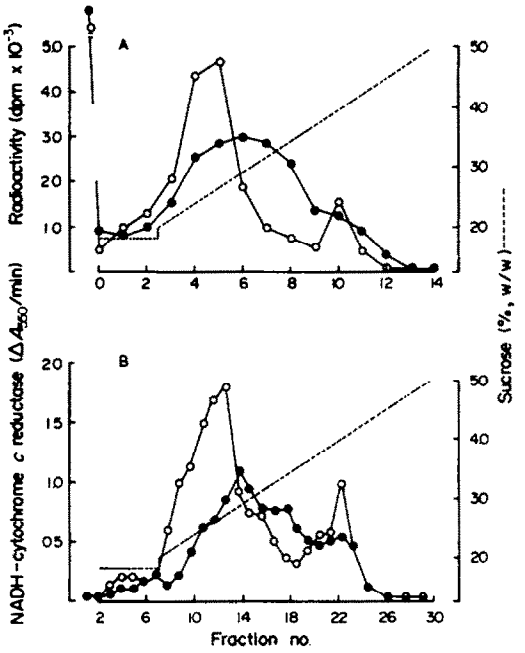

Fig. 3. Effect of Mg^{2+} concentration on distribution of endoplasmic reticulum membranes on sucrose gradients. A. Distribution of radioactivity; B. distribution of NADH-cytochrome *c* reductase activity; ○ 0.1 mM Mg^{2+}; ● 3 mM Mg^{2+}

condition the peaks of radioactivity and NADH-cytochrome *c* reductase activity at 1.11–1.12 g/cm³ were shifted to form broader peaks at 1.12–1.14 g/cm³. This shift is typical of ER membranes and it is due to the retention of larger numbers of ribosomes on the membrane [12].

Some of the chemicals which had proved effective in reducing membrane damage during cell fractionation were tested as inhibitors of PLase D activity. A cell-free preparation from aleurone tissue of 14 hr embryoectomized seed gave an activity of 8.5 μmol/30 min/30 seeds. PCHOL, G-1-P or a mixture of the two compounds were only partly effective as inhibitors of this activity (Table 4). Surprisingly, KF was almost inactive while *p*CMB was a strong inhibitor.

DISCUSSION

The results in this paper corroborate our earlier suspicions about the extent of damage to aleurone cell membranes during subcellular fractionation. In the absence of inhibitors, only small quantities of ER membranes equilibrated at the expected density on sucrose gradients. Most of the damage occurred during tissue homogenization and prior to gel filtration of the homogenate. Little further degradation of the membranes occurred during the subsequent centrifugation on sucrose gradients. Despite the well-documented Ca^{2+}-dependence of PLase D [6], quite high concentrations of EDTA were insufficient to protect against the activity of the enzyme and those of other possible lipases. A similar problem was encountered by Scherer and Morre [7]. These findings call into question some of the results of earlier sub-cellular studies on cereal aleurone tissue and other plant tissues containing high PLase D activities [13].

Among the inhibitors which were effective in protecting the aleurone tissue membranes, PCHOL was effective at reasonably low concentrations. It is probably the most useful of the inhibitors examined. Choline and ethanolamine were also effective, but high concentrations were needed. At the required concentration, ethanolamine also caused considerable problems due to its perturbation of the pH. *p*CMB was particularly effective when used as part of a cocktail of inhibitors, but its use would be disadvantageous if enzyme studies were to be carried out on the sub-cellular fractions. These results emphasize above all the importance of using suitably protective media for subcellular studies on plant tissues. Choline and PCHOL can be recommended for most applications, but the best cocktail of inhibitors should be sought for each experimental tissue.

Loss of radiolabelled choline from PC, its inhibition by various choline analogues and the presence of PLase D activity in the aleurone tissue, suggest that the enzyme was principally responsible for the observed membrane damage. The finding that the *in vitro* effects of the inhibitors on the enzyme activity were not always the same as their protective action during membrane isolation does not detract from this conclusion. Any difference can probably be explained by the fact that the enzyme's activity and substrate specificity are dependent upon its degree of purity and the physical state of its substrate [10, 14].

The mechanism of PLase D inhibition by PCHOL is unknown. It is likely, however, that it works by a similar

Table 4. Inhibition of phospholipase D activity

Inhibitor	Enzyme activity (μmol/30 min/30 seeds)	Inhibition (%)
None	8.2 ± 1.7(3)	—
10 mM G-1-P	4.8 ± 0.9(3)	42
10 mM PCHOL	6.1 ± 1.5(3)	26
25 mM PCHOL	5.1 ± 1.5(5)	38
25 mM PCHOL + 10 mM G-1-P	4.7 ± 0.7(3)	43
10 mM KF	7.9 ± 1.0(4)	4
0.1 mM *p*CMB	0.2 ± 0.4(4)	98
1 mM *p*CMB	0 (4)	100

The experiment was carried out on tissue from 30 embryoectomized seeds incubated for 14 hr. The values for enzyme activity are means ± s.d. of the number of experiments in parentheses.

mechanism to that of choline, that is as a substrate analogue which binds to the reaction centre of the enzyme [10, 11]. Inhibition by other analogues such as AMO 1618 [15] supports this view. Since PCHOL is a major constituent of the cell sap [16 17], it may act to control PLase D activity *in vivo*. Cell sap components which are inhibitors of PLase D have, in fact, been reported [18, 19]. During tissue homogenization these compounds would be inadvertently diluted in the medium, thereby initiating enzyme activity. This interesting possibility warrants further investigation.

EXPERIMENTAL

Reagents. General reagents were purchased from BDH Ltd. Cytochrome *c*, NADH and digitonin were bought from Sigma Chemical Co. and Sepharose 4B-CL was from Pharmacia Ltd. [Me-^{14}C]Choline was obtained from Amersham International plc. PCHOL, obtained as its Ca^{2+} salt from Sigma, was converted to the K^+ salt on a column of IRC-50 (H^+) ion-exchange resin. The K^+ salt was stored for not more than 1 day before use.

Sterilization and incubation of plant material. Soft-grained winter wheat, cv Flanders, was used in batches of 100 seeds weighing 52 g. The embryo ends of the seeds were removed by a transverse cut with a scalpel, and the embryoectomized seeds were sterilized and incubated in the dark at 25° as described previously [20]. At the end of the incubation, the embryoectomised seeds were dissected to obtain their aleurone layers (aleurone cells + pericarp).

Radiolabelling of phospholipids. Aleurone layers from either 50 or 100 seeds were incubated for 30 min at 25° in 10 (15) ml 50 mM Tris–maleate buffer, pH 6.7, containing 2.5 (5.0) μCi [Me-^{14}C]choline (58 μCi/mmol). At the end of this incubation the tissue was removed from the radioactive medium by filtration and washed × 3 with H_2O.

Sub-cellular fractionation. Washed aleurone tissue was homogenized using motorized razor blades by a procedure adapted from refs [21] and [8]. The isolation medium contained 18.5% (w/w) sucrose, 10 mM KCl, 0.1 mM $MgCl_2$, 3 mM EDTA and 50 mM Tris–maleate buffer, pH 7.5. Inhibitors were added as recorded in the Results section. The resulting homogenate was quickly filtered through 64 μm polyester mesh and the residue on the mesh was washed with 3 × 1 ml isolation medium. This residue was collected as the cell debris fraction. The combined filtrate was centrifuged for 10 min at 800 *g* and the resulting pellet was collected. The supernatant was applied to a column of Sepharose 4B-CL (1.5 × 26 cm) prepared in isolation medium and isolation medium was used to elute the column. Turbid fractions, which were eluted at the void vol. and which contained particulate material, were pooled (3.6 ml) and loaded onto 14.4 ml isolation medium containing a continuous gradient of 20–50% (w/w) sucrose in a polycarbonate centrifuge tube. The sample was then centrifuged for 4 hr at 29 000 rpm. A lipid-containing pad, which collected at the top of the tube, was carefully removed with either petrol (bp 40–60°) for lipid analysis or H_2O for enzyme assay. The remaining gradient was fractionated using an ISCO 640 fractionator.

Extraction and analysis of lipids. Total lipid was extracted from whole tissue, tissue homogenates, cell debris or 800 *g* pellets with hot H_2O-satd 1-butanol. The lipid was then purified by chromatography on Sephadex G-25 and fractionated by CC and TLC on silicic acid. These methods have been described previously [4]. Radioactivity in the fractions was determined by liquid scintillation counting (LSC). Radioactivity in the particulate fractions from Sepharose chromatography and sucrose gradient centrifugation was located exclusively in PC. This obviated the need for extraction and purification of the lipids, and radioactivity in these fractions was determined directly by LSC.

Enzyme assays. NADH–cytochrome *c* reductase was assayed by the method of ref. [12]. The assay mixture contained 0.06 mM cytochrome *c*, 0.6 mM NADH and 15 mM KCN in 50 mM K Pi buffer, pH 7.2. Cytochrome oxidase was assayed by the method of ref. [22]. The assay mixture contained 0.1 mM reduced cytochrome *c* and 0.2% digitonin in 50 mM K Pi buffer, pH 7.2.

PLase D was assayed using a method based on those of refs. [11] and [23]. Aleurone tissue was homogenized for 2 min in H_2O using a Polytron homogenizer. The homogenate was centrifuged for 5 min at 800 *g* and the supernatant was used for the assay. The assay mixture contained 1 mM egg lecithin, 0.5 mM SDS, 40 mM $CaCl_2$ and 80 mM Na OAc buffer, pH 5.6. Inhibitors were added as recorded in the Results. After incubation for 30 min at 28° the reaction was stopped by adding 0.2 ml 75% TCA. The reaction tubes were stored on ice for 1 hr and then centrifuged for 5 min at 5000 *g*. Choline was determined in the supernatant. Zero-time controls were included with each assay.

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