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PURIFICATION OF GIBBERELLIC ACID-INDUCED LYSOSOMES FROM WHEAT ALEURONE CELLS

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SUMMARY

Using isopycnic density gradient centrifugation, lysosomes were concentrated in a single region of a sucrose–Ficoll gradient (ρ = 1·10 g cm^{-3}), well separated from most other cell organelles. Gibberellic acid-induced lysosomes were found to be rich in α-amylase and protease but not ribonuclease. The lysosomal band also contained a majority of the $NADH_2$-cytochrome c reductase, a marker enzyme for endoplasmic reticulum, found in the gradient. Examination of electron micrographs revealed that a purified band of lyosomes contained at least 3 vesicle types, ranging in size from 0·1 to 0·5 μm. The significance of these findings to proposed mechanisms of action of gibberellic acid is discussed.

INTRODUCTION

The mechanism by which the acid hydrolases induced by gibberellic acid (GA_3) are released from cereal aleurone cells has recently received attention (Jones, 1972; Varner & Mense, 1972; Gibson & Paleg, 1972). Investigations with wheat aleurone revealed that α-amylase and protease were sedimentable by standard centrifugation techniques, thus indicating a particulate rather than a soluble mode of enzyme release (Gibson & Paleg, 1972, 1975). In a series of publications dealing with barley aleurone tissue, Jones (1972), Chen & Jones (1974*a*, *b*) and Jones & Chen (1976) were unable to obtain comparable evidence and concluded that the hydrolases formed in barley in response to GA_3 had a soluble rather than a particulate release mode. However, Firn (1975), also working with barley aleurone, was able to confirm the wheat results, and he and Gibson & Paleg (1975) indicated several of the difficulties involved in isolating particulate enzymes from aleurone cells.

Our original observations of pH optima, sedimentability and structural latency, prompted the suggestion that the hormone-induced enzymes were lysosomal, i.e. membrane-enclosed (Gibson & Paleg, 1972). However, although subcellular particles designated lysosomes have been identified in a number of plant tissues (Matile 1968; Semadeni, 1967; Pitt & Galpin, 1973), none has been shown to be associated with hormone action and few can be considered analogous to animal lysosomes (Adams & Novellie, 1975*a*, *b*; Parish, 1975*a*, *b*). Thus, since both the particulate nature of the aleurone enzymes, and the very existence of lysosomes in plants, are controversial topics, it was desirable to examine the nature of our enzyme-containing particles more

closely. This paper reports the results of attempts to purify the organelles containing the particulate α-amylase and protease of wheat aleurone cells.

MATERIALS AND METHODS

Aleurone tissue was prepared from wheat seeds (cv. Olympic), using techniques detailed previously (Gibson & Paleg, 1972, 1975), and incubated for 24 h in the presence or absence of GA_3 (1 μg/ml). At the end of the incubation period, the tissue (1 g) was washed with 2 × 100 ml of distilled water, 10 ml of 10 mM EDTA for 5 min, and finally rinsed with 2 × 100 ml of distilled water. All subsequent operations were carried out at 2 °C. The chilled tissue (1 g) was homogenized with either a mortar and pestle or an Ultra Turrax model TP/180 in 10 ml of medium containing 0·4 M sucrose, 50 mM Tris-HCl (pH 7·0), 0·1 % bovine serum albumin, 10 mM KCl, 1 mM EDTA and 0·1 mM $MgCl_2$. The cell homogenate was filtered through 2 double layers of cheesecloth and centrifuged at 1000 g for 10 min. The resulting supernatant was recentrifuged at 60000 g for 30 min, and the pellet was resuspended in 1 ml of medium containing 0·4 M sucrose, 10 mM Tris-HCl (pH 7·0) and 1 mM EDTA with a plastic piston device having a 0·1-mm clearance. The resuspended pellets from 4 g of tissue were mixed and layered on the top of density gradients.

The gradients were prepared, using a peristaltic pump device capable of coping with viscous Ficoll solutions. Unless otherwise stated, all gradients contained 10 mM Tris-HCl (pH 7·0) and 1 mM EDTA, and were prepared at least 12 h in advance. All gradients were 30 ml in volume, including the load, and were poured into cellulose nitrate tubes made for use in the Beckman SW 25.1 rotor. Exact composition of gradients and length of time in the centrifuge varied and are explained in the text.

At the end of the centrifugation period, fractions (1 ml) were collected by means of an ISCO density gradient fraction collector at a flow rate of 2·5 ml/min. Density gradient profiles were obtained by recording the absorbency of material from the gradient at 280 nm on an ISCO density recorder set at 2·5 units full-scale absorbance. The collected fractions were immediately chilled and enzyme activity was measured as soon as possible.

Enzyme assays

α-Amylase (*EC* 3.2.1.1). When this was the only enzyme to be assayed, each fraction from the gradient was collected in tubes containing 4 ml of 10 mM calcium acetate solution. When other enzymes were also assayed a 0·2-ml sample from each gradient fraction was placed in a separate tube containing 0·8 ml of 10 mM calcium acetate. The diluted α-amylase samples were then made 0·1 % with respect to Triton X-100 and heated for 10 min at 70 °C before measurement of enzyme activity by the method detailed earlier (Gibson & Paleg, 1975).

Acid ribonuclease (*EC* 2.7.7.16). Samples (0·2 ml) from each of the density gradient fractions were diluted with 0·8 ml of 0·1 M acetate-Tris buffer (pH 5·0) and the diluted enzyme fractions made 0·1 % with Triton X-100. The depolymerization of yeast RNA was determined by the method of Wilson (1963).

Acid protease (*EC* 3.4.4). Samples (0·5 ml) from each of the density gradient fractions were diluted with 0·5 ml of 0·1 M acetate-Tris buffer, pH 4·8, and made 0·1 % with Triton X-100. Hydrolysis of the wheat storage protein, gliadin, was assayed by the method of Jacobsen & Varner (1967).

Cytochrome c oxidase (*EC* 1.9.3.1). This enzyme was assayed according to Simon (1958), using 25–30 μl of enzyme taken directly from the gradient fractions.

Catalase (*EC* 1.11.1.6). Assayed by the method of Maehly & Chance (1954), using 25–50 μl of enzyme taken directly from the density gradient fractions.

$NADH_2$ cytochrome c reductase (*EC* 1.6.2.1). The reduction of cytochrome c was followed in a Unicam SP1800 recording spectrophotometer at 550 nm and 25 °C. The final reaction volume was 1·2 ml and contained 25 μM $NADH_2$, 25 μM cytochrome c, 0·167 mM KCN, 50 mM KH_2PO_4 (pH 7·6) and 5–50 μl of enzyme taken directly from the gradient fractions.

Presentation of data

Unless otherwise stated, all enzyme data are presented as arbitrary units, where one unit equals 1 % of the total activity found in that particular density gradient.

Electron microscopy

Gradient fractions that corresponded to visible bands were combined and fixed in 2 % (v/v) glutaraldehyde in phosphate buffer (pH 7·5) for 8 h at 5 °C. The samples were then centrifuged at 60000 **g** for 30 min and the resulting pellets washed with 0·4 M sucrose solution prepared in phosphate buffer (pH 7·5). The samples were centrifuged for a second time and the resulting supernatant discarded.

The pellets were resuspended in one drop of 2 % (w/v) agar at 45 °C. The cooled agar-organelle mixture was easier to handle than loose pellets and the organelles were postfixed in 2 % (w/v) osmium tetroxide in 0·1 M phosphate buffer, pH 7·0, for 2 h. The pellet fractions were dehydrated in an ethanol series and embedded in Araldite. Ultrathin sections were cut on a Si-Ro-Flex ultramicrotome, mounted on copper grids and examined, after counterstaining in lead citrate, in a Siemens electron microscope.

Incorporation of [^{14}C]*lysine*

Two 1-g samples of aleurone tissue that had been incubated for 23 h with or without GA_3 were incubated for a further 2 h with 1 μCi of DL-[1-^{14}C]lysine monohydrochloride (specific activity 314 μCi/mg, The Radiochemical Centre Ltd, Amersham, England). The tissue was washed in copious amounts of distilled water and homogenates were prepared by the method described above. The organelles were separated on a sucrose-Ficoll gradient and samples of the resulting fractions were mixed with Bray's solution (Bray, 1960) and radioactivity determined in a Packard Model 'Tri-Carb' scintillation counter.

RESULTS

Repeated experiments with density gradients composed of sucrose failed to yield reproducible results with aleurone cell organelles in the 60000 **g** pellet. Ficoll gradients, although difficult to prepare due to the viscosity of concentrated solutions, were far more reliable and the results of a typical experiment using sucrose–Ficoll are shown in Fig. 1. A distinct peak of α-amylase activity, associated with bands 2 and 3, corresponded to a density of 1·10 g cm^{-3}.

All features of the gradient were found to be essential for good particle separation. Without the overlay of 6 ml of 20% sucrose, most of the organelles failed to penetrate the gradient and without 1 mM EDTA, severe aggregation of organelles occurred probably due to trace amounts of calcium (Gibson & Paleg, 1975). Centrifugation for longer periods (up to 19·5 h) failed to achieve significantly better separation of the visible bands in the gradient.

Earlier experiments (Gibson & Paleg, 1972) indicated that while α-amylase and protease were particulate enzymes, the exact nature of ribonuclease was in doubt. It was therefore important to establish the distribution of these 3 GA_3-induced hydrolytic enzymes in the various fractions of the gradient. Most of the ribonuclease activity was associated with the supernatant (top) of the gradient, although a significant proportion was found down the length of the gradient (Fig. 2). Because no peak of ribonuclease activity was found associated with any particular band, it was concluded

that a majority of this enzyme was either cytoplasmic or only poorly restricted to the organelles. Acid protease, on the other hand, showed an identical distribution pattern to that of α-amylase, with a majority of the enzyme detected in the region of bands 2 and 3. It seemed likely, therefore, that these 2 enzymes were either associated with the same particle or in different particles of the same density which the gradient was unable to resolve.

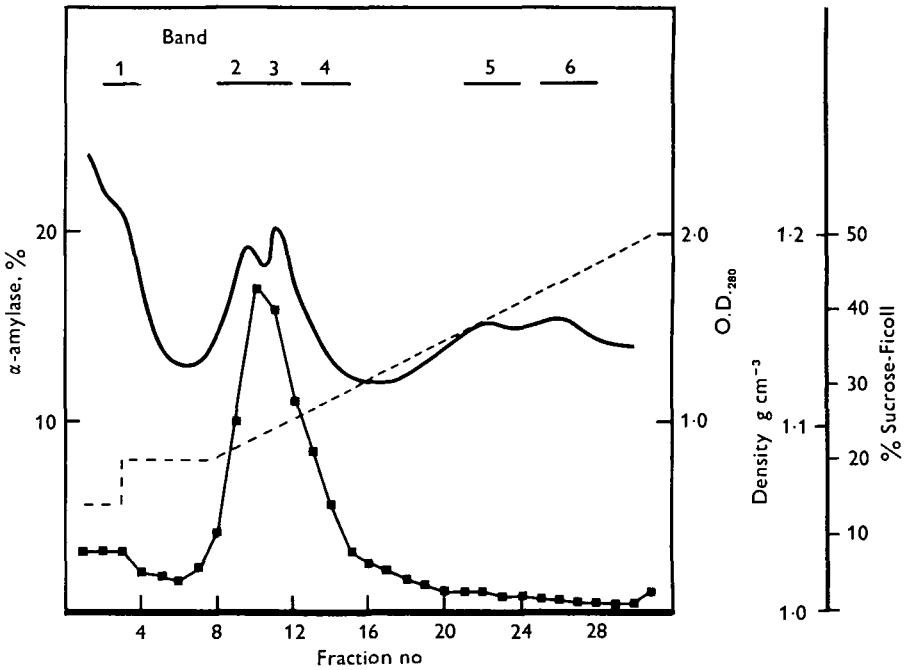

Fig. 1. Distribution of α-amylase on a linear sucrose:Ficoll density gradient (20% sucrose to 20% sucrose + 30% Ficoll) after centrifugation at 20000 g for 4 h at 5 °C in a Beckman 25.1 rotor. All gradient solutions contained 1 mM EDTA and 10 mM Tris-HCl (pH 7·0). The 2-ml load contained organelles isolated from aleurone which had been treated with GA_3 (1 μg/ml) for 24 h at 30 °C. Enzyme activity is expressed in arbitrary units where 1 unit equals 1% of the total activity detected on the gradient. ■——■, α-amylase; ———, O.D. 280 nm; — — —, % sucrose-Ficoll density.

The various bands observed in the density gradients were assayed for enzymes known to be associated with particular organelles (Fig. 3). $NADH_2$ cytochrome c reductase, a marker enzyme for endoplasmic reticulum (ER), was almost entirely located in the region of bands 2 and 3, as was α-amylase. The resolution of bands 5 and 6 was poor, with band 5 containing a majority of the cytochrome c oxidase activity. It was concluded that this band contained mainly mitochondria. Catalase activity, on the other hand, was equally distributed between band 1 and bands 5 and 6. Band 4, a shoulder of band 3, contained appreciable amounts of both α-amylase and cytochrome c reductase. Other attempts were made to separate α-amylase and cytochrome c reductase activities, using both shallow and discontinuous gradients. However, they were unsuccessful since no clear separation of the 2 enzymes was achieved in

either gradient. All these results suggested that the α-amylase-containing particles were either ER vesicles or particles that were of similar density.

Although cytochrome c reductase has been reported to be associated with the ER (Moore, Lord, Kagawa & Beevers, 1973), it was desirable to establish the identity of this region of the gradient by other means. Since the ER is a site of protein synthesis, it was reasoned that this region of the gradient should incorporate ^{14}C-amino acids. Accordingly, samples of aleurone tissue that had been incubated for 23 h with or without GA_3 were incubated a further 2 h with 1 μCi [^{14}C]lysine, and the tissue homogenates subjected to density gradient centrifugation.

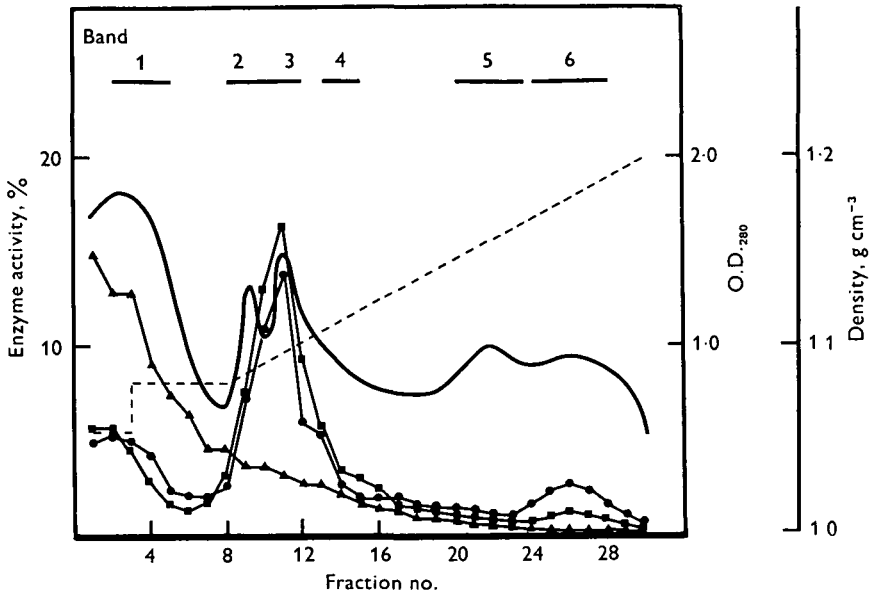

Fig. 2. Distribution of α-amylase, protease and ribonuclease on a linear sucrose:Ficoll gradient. All conditions as described for the previous figure. ■——■, α-amylase; ●——●, protease; ▲——▲, ribonuclease; ———, O.D. 280 nm; — — —, density.

The results are presented in Fig. 4, and several points can be noted. Although there is a large peak of radioactivity in the ER region of the control gradient (Fig. 4A), this peak is very much reduced in the gradient containing organelles from GA_3-treated tissue (Fig. 4B). The reduced level of [^{14}C]lysine incorporation in the GA_3-treated tissue was probably due to isotope dilution by proteolysis (Varner, Ram Chandra & Chrispeels, 1965). A second major peak of radioactivity was also observed in control tissue gradients in the mitochondria-glyoxysome region which was not observed in the GA_3-treated tissue gradients.

α-Amylase was not detected in the control gradients (Fig. 4A) but was presented as a single peak on the gradient obtained with organelles from GA_3-treated tissue (Fig. 4B). Furthermore, the α-amylase peak, as usual, corresponded exactly to the peak obtained for cytochrome c reductase (Fig. 4B). Finally, the amount of

cytochrome c reductase measured in the ER region of both gradients was identical (6·5 units). Thus, it would appear that GA_3, under the conditions of this experiment, did not cause an increase in ER, as measured by the marker enzyme for this fraction of the cell contents.

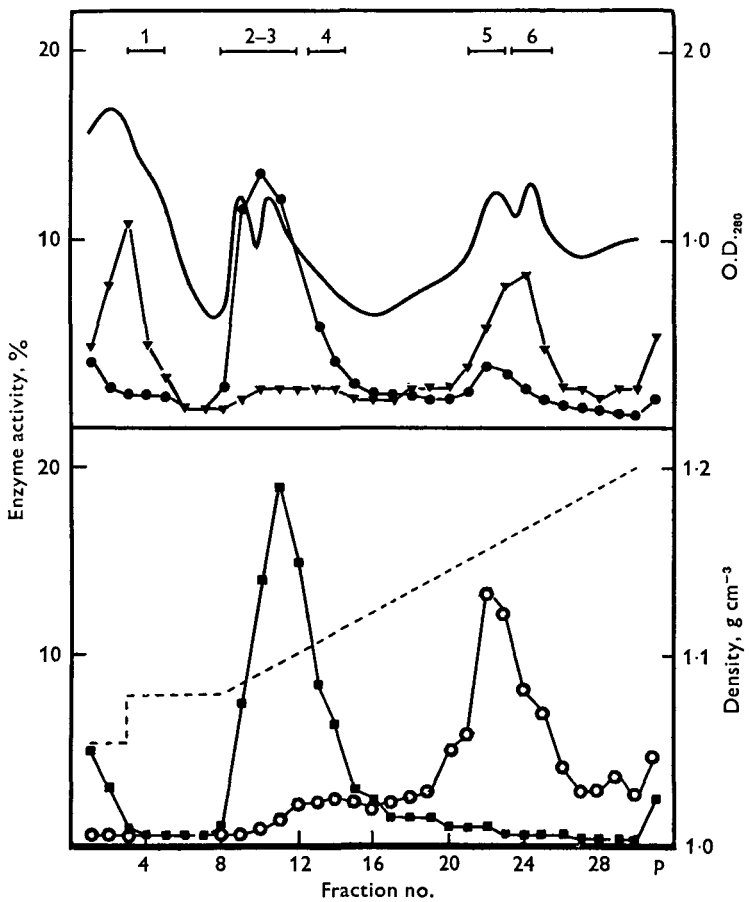

Fig. 3. Comparative distribution of various organelle marker enzymes on a linear sucrose:Ficoll gradient after ultracentrifugation. All conditions as described for Fig. 1. ■——■, α-amylase; ●——●, $NADH_2$-cytochrome c reductase; ▼——▼, catalase; ○——○, cytochrome c oxidase: ———, O.D. 280 nm; — — —, density.

Examination of electron micrographs of the ER-lysosome region of density gradients indicated that the fractions obtained were rather heterogeneous even though biochemical analysis had indicated a fair degree of marker enzyme purity (Fig. 3). An attempt was made to purify the lysosomes still further by removing bands 2, 3 and 4 from a gradient similar to that shown in Fig. 1, and recentrifuging them on a shallow gradient (Fig. 5). This resulted in the formation of only one major band which contained both α-amylase and cytochrome c reductase activity. This band was rich in 3 types of organelles; large vesicles (0·5 μm) often containing small electron-dense

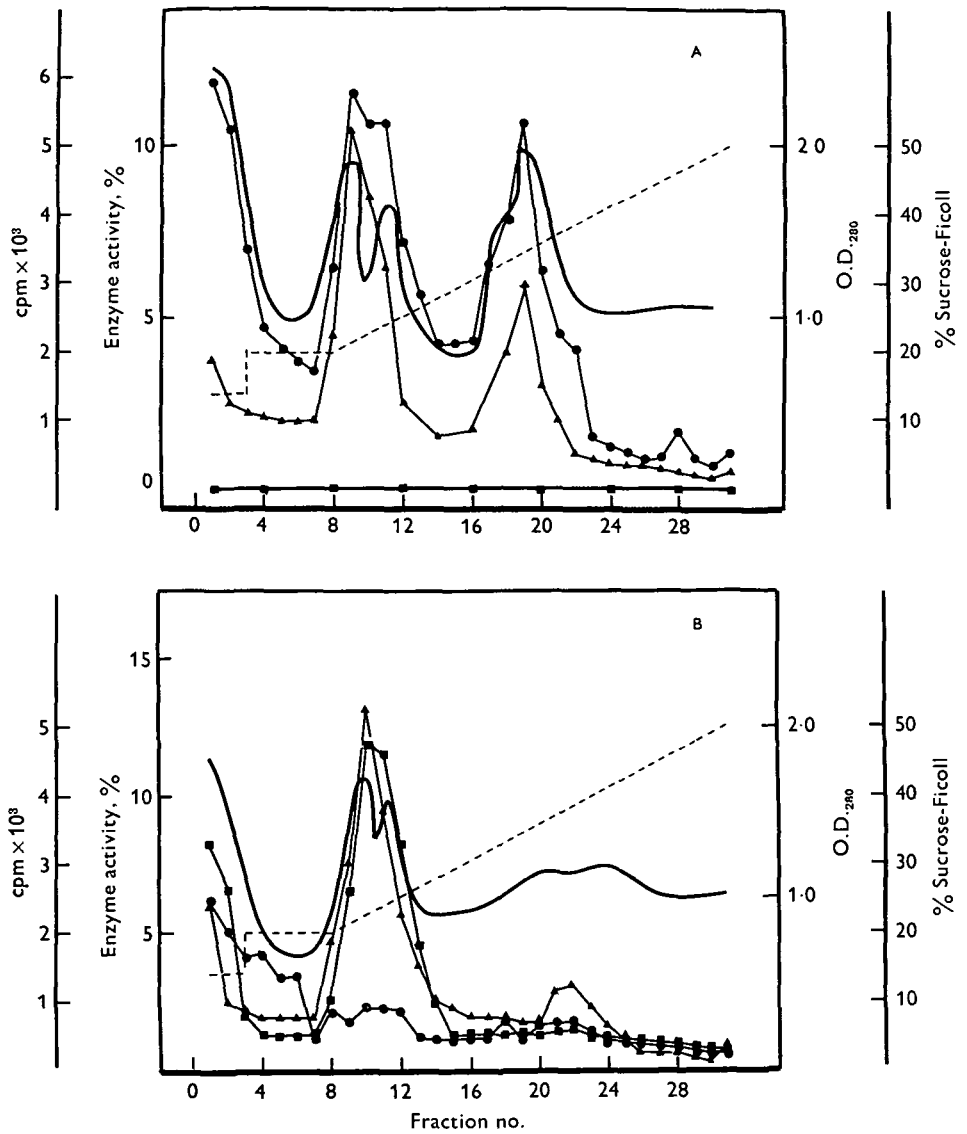

Fig. 4. Incorporation of [^{14}C]lysine into organelles isolated from control (A) or GA_3-treated (B) tissue and separated on a linear gradient as described in Fig. 1. ■——■, α-amylase; ▲——▲, $NADH_2$-cytochrome c reductase; ●——●, radioactivity; ———, O.D. 280 nm; — — —, density.

bodies; vesicles of smaller (0·2–0·4 μm), irregular size with double membranes, and smaller (0·1–0·2 μm), slightly electron-dense vesicles (Fig. 6). It was obvious that complete purification of the lysosomes had not been achieved.

DISCUSSION

Considering that isopycnic centrifugation is a well established technique for organelle isolation (Tolbert, 1973), the degree of difficulty experienced in obtaining

satisfactory separation of lysosomes from other organelles was surprising. Although the poor results obtained in preliminary experiments could be attributed to the presence of calcium (Gibson & Paleg, 1975), the indifferent resolution of organelles on sucrose density gradients, even in the presence of EDTA (data not shown), was disappointing Similar difficulties have been reported by Parish (1971) but the reasons are far from clear.

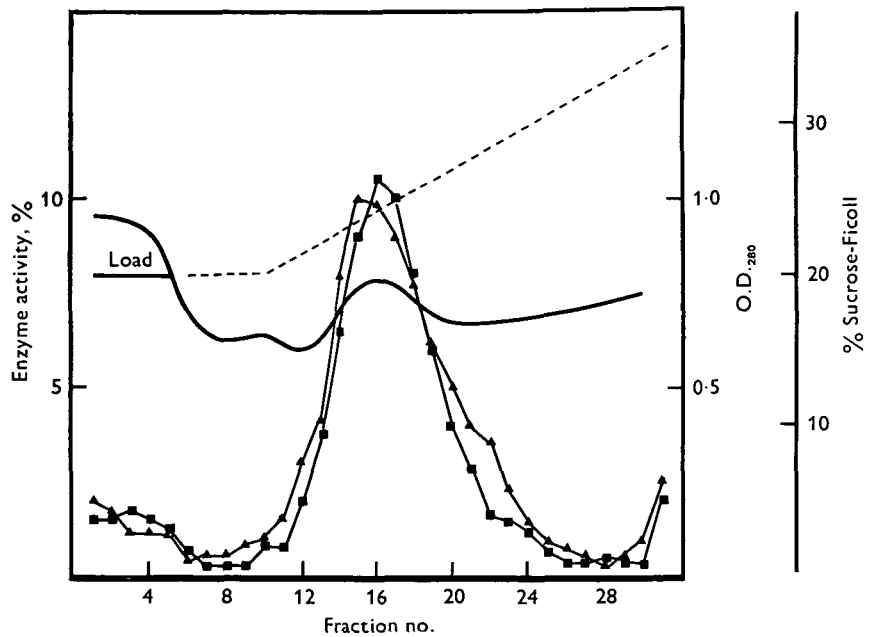

Fig. 5. Purification of a lysosome-rich peak obtained from a density gradient similar to that shown in Fig. 1 (including bands 2, 3 and 4) by a second centrifugation on a linear gradient ranging from 20 % sucrose to 20 % sucrose + 15 % Ficoll. The second centrifugation was for 4 h at 5 °C at a speed of 25000 rev/min in a Beckman SN 25.1 rotor. ■——■, α-amylase; ▲——▲, $NADH_2$-cytochrome c reductase; ———, O.D. 280 nm; — — —, density.

The separation of organelles was greatly improved when Ficoll was used as the solute (Fig. 1) as it had also been for Pitt & Galpin (1973) in their separation of lysosomes from potato shoots. Unfortunately, however, Ficoll seriously interferes with reagents used to measure protein, making accurate determinations impossible, and it may also affect the organelles as well as their separation (e.g. Tolbert (1973) reported serious losses of microbody marker enzymes when Ficoll was used as the solute).

Enzyme analysis of the sucrose-Ficoll linear density gradients (Fig. 3) revealed that excellent separation of lysosomes from other major organelles had been achieved. Cytochrome c oxidase, a marker enzyme for mitochondria, was located in band 5 and, to a lesser extent, band 6. Electron micrographs confirmed that band 5 contained almost pure mitochondria. The apparent buoyant density of mitochondria in the Ficoll gradient was 1·150 g cm^{-3}, which is lower than that (1·20 g cm^{-3}) normally

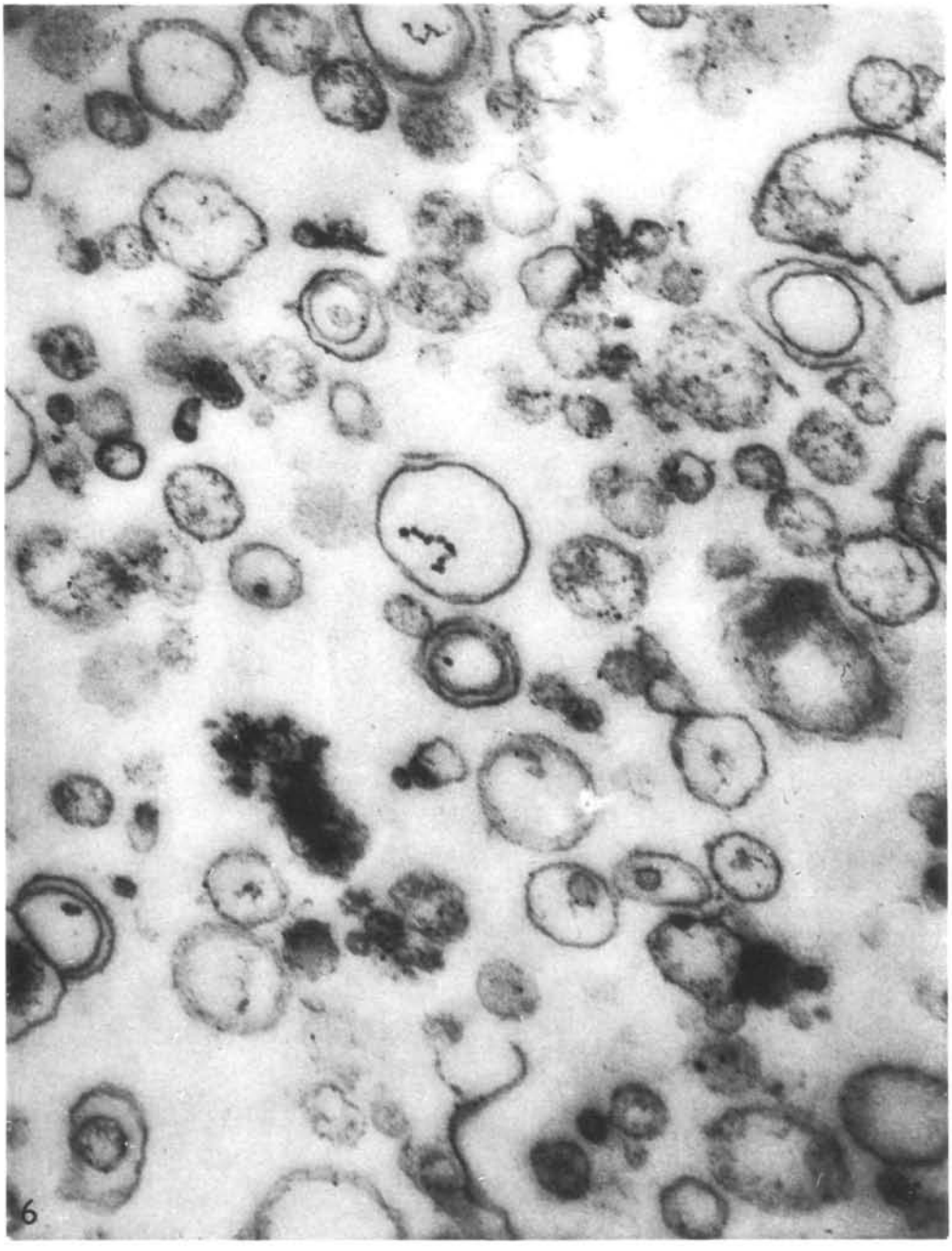

Fig. 6. Electron micrograph of lysosome-rich peak shown in Fig. 5. ×40000.

reported for mitochondria (Rocha & Ting, 1970). This is almost certainly due to the use of Ficoll, which does not penetrate readily into organelles and slows the centrifugal movement of organelles in the gradient (Pitt & Galpin, 1973).

The distribution of the microbody (peroxisome, glyoxysome) marker enzyme, catalase, is puzzling. It was found associated with both band 1 and band 6. As microbodies are reported to have a density of 1·25 g cm^{-3} (Breidenbach & Beevers, 1967), which should locate them immediately below mitochondria (band 6), the catalase activity at the top of the gradient ($\rho = 1{\cdot}06$) is difficult to explain. Tolbert *et al.* (1969) and Parish (1971) have reported similar observations. The identity of band 1 remains obscure but the organelles in band 6 have tentatively been called microbodies.

The lysosome region of the density gradient (bands 2 and 3) is not only rich in α-amylase but also contains most of the protease activity. Nakano & Asahi (1974) isolated a membrane component from the microsomal fraction from peas rich in acid protease. Ribonuclease, on the other hand, must be considered to be either a soluble enzyme or, if particulate, very permeable with respect to the particle membrane. Reports of ribonuclease activity associated with plant lysosomes (Matile, 1968; Pitt & Galpin, 1973) must be considered in the light of the work of Matsushita & Ibuki (1960) who showed that most of the particulate ribonuclease in peas was associated with ribosomes attached to membranes. Furthermore, Hirai & Asahi (1973) have shown that ribonuclease associated with pea microsomes was only loosely attached to the membranes. It is also possible that tissue treated with hormone for 24 h does not have a sufficiently high percentage of newly formed ribonuclease to identify it adequately as particulate.

The enzyme-containing particles from wheat aleurone lysosomes appear to be associated with the ER, as measured by cytochrome c reductase activity (Figs. 3–5) and by incorporation studies with [^{14}C]lysine (Fig. 4). Thus, the site of synthesis of GA_3-induced α-amylase and protease is probably the ER, although direct proof of this proposal was not obtained in this study. Measurements of amounts of ER in GA_3-treated and control tissue, by assaying cytochrome c reductase in the ER region of gradients, failed to reveal a significant difference between treatments. Reports by Jones (1969*a*, *b*) and Vigil & Ruddat (1973) of massive proliferation of RER in response to GA_3 treatment in barley aleurone cells must be questioned. Since cytochrome c reductase has definitely been shown to be associated with ER in animals (Goldstone *et al.* 1973) and plants (Moore *et al.* 1973), it is considered that this enzyme is a good measure of ER level when assayed from the proper region of a density gradient. It may well be that the cytological observations of apparent ER proliferation (Jones, 1969*a*, *b*; Vigil & Ruddat, 1973) were actually an unmasking of pre-existing ER as the aleurone cells expand during imbibition and the aleurone grains and spherosomes reduce in number in response to GA_3 (Paleg & Hyde, 1964; Jones 1969*a*, *b*). A similar conclusion has been reached by Laidman, Colbourne, Doig & Varty (1973).

The lysosomal region of the density gradient (bands 2 and 3) was calculated to have a buoyant density of about 1·10 g cm^{-3} which is in close agreement with values

obtained for lysosomes from other plant material. For example, Matile (1968) isolated a heavy lysosome fraction with an apparent density of 1·11 g cm^{-3} which was rich in cytochrome c reductase activity from corn roots. Semadeni (1967) isolated vesicles from tobacco which appeared to be ER-derived and contained α-amylase activity at a position in density gradients corresponding to a density of 1·07 g cm^{-3}. More recently Pitt & Galpin (1973) found 2 populations of lysosomes (ρ = 1·10 and 1·07 g cm^{-3}) in organelle preparations from potato shoots which were rich in RNA and composed of a heterogeneous collection of vesicles, many of which contained double membranes. Finally, Hirai & Asahi (1973) critically examined Matile's report (1968) of several populations of lysosomes in plant roots. They demonstrated that, although density gradient experiments, as run by Matile, indicated 2 lysosomal populations (one heavy, ρ = 1·145, and the other much lighter), since neither population could be separated from cytochrome c reductase, they concluded that both populations were ER-derived.

Attempts to purify hydrolase-containing particles in the present study were only partially successful, in that the purest fraction contained at last 3 different vesicles. Whether the vesicles are true lysosomes or ER vesicles derived during homogenization, as suggested by Jones & Chen (1976) is not yet clear. From a functional stand point, at least, the particulate α-amylase and proteinase of wheat aleurone conforms with the established concepts of lysosomal enzymes. Our data are consistent with the suggestions (Vigil & Ruddat, 1973; Chen & Jones, 1974*a*, *b*) that the site of α-amylase and proteinase synthesis is probably the ER and thus the synthesis of these enzymes is subject to the same controls as the synthesis of other extracellular enzymes.

We think that the present results indicate a particulate, rather than soluble mode of enzyme release; in fact, one may ask why the cell would find it advantageous to synthesize and pass extracellular hydrolytic enzymes into the lumen of the ER only to have them diffuse through the cytosol prior to release (Jones & Chen, 1976). In addition, the very high percentage (> 80%) of α-amylase found as particulate enzyme in GA_3-treated wheat aleurone cells (Gibson & Paleg, 1975) was uncorrected for vesicle breakage during homogenization, or incomplete inactivation of α-amylase retained in the cell walls. Both factors suggest an even higher actual proportion of particulate enzyme in wheat aleurone.

That these hormonally controlled enzymes are also secreted makes the response additionally interesting. Although lysosomes have been found in most animal tissues (Dingle & Fell, 1969), few lysosomal enzymes are actively secreted from the cells. However, the few cases that have been reported also appear to be under hormonal control. Secretion of protease by bone cells, for example, is controlled by parathyroid hormone and the secretion of a similar protease from thyroid cells is stimulated by TSH (Dingle & Fell, 1969). None of the other reported cases, though, match the aleurone response in terms of speed and intensity of the effect, and, thus, the GA_3-induced synthesis and secretion of hydrolytic enzymes by cereal aleurone cells appears yet again to be unique.

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