CHAPTER 13

Lysosomes

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I. The Lysosome Concept

During the early studies on the hydrolases uricase and acid phosphatase, some workers (Palade, 1951; Berthet and de Duve, 1951; Schein *et al.*, 1951; Schneider and Hogeboom, 1952) found that if tissues were homogenized and the homogenates were subjected to differential centrifugation, then these enzymes appeared in the mitochondrial fractions. Equally, when similar experiments were performed by other workers (Tsuboi, 1952; Novikoff *et al.*, 1953) these two enzymes appeared to be associated with the microsomal fraction. It was the discrepancy between the various groups of results that enabled de Duve and his collaborators (de Duve *et al.*, 1953; Appelmans *et al.*, 1955) ultimately to define the lysosomes. By adjusting the methods of centrifugation, it was possible to show that the mitochondrial fraction from rat liver consisted of two sub-fractions which were termed heavy and light mitochondria. The light mitochondrial fraction was found to contain the highest concentration of acid phosphatase and yet corresponded to only approximately 4% of the total homogenate nitrogen. Further investigations showed the light mitochondrial fraction to contain also high concentrations of cathepsin, acid ribonuclease, acid deoxyribonuclease and β-glucuronidase. This list has now been extended to include lipase, phospholipase c, α-glucosidase, arylsulphatases A and B, α-manosidase, β-galactosidase and β-N-acetyl-glucosaminidase. When these particles were isolated in 0·25M sucrose, they

appeared from biochemical evidence to be intact since relatively little hydrolase activity could be found in the supernatant fraction. However, if the particles were treated in a number of ways (Table I) then the enzymes appeared in the supernatant fraction and were no longer bound to the particles.

TABLE I

Treatments affecting the structure of lysosomes

Sonication	CCl_4
Inadequate osmotic protection	Waring blender
Lecithinase	Freezing and thawing
Detergents	Fat solvents
Protease	Autolysis (acid pH and high temperature)

Towards the end of the initial studies, there emerged some particular features concerning this group of particles. Firstly, the particular hydrolases studied appeared to be associated with the same group of particles. Secondly, these hydrolases all had an optimum activity at about pH 5. When initially isolated, there was virtually no soluble enzyme activity, a feature termed "latency". This enzyme activity, however, could be made available by suit-

TABLE II

Morphological variation in lysosomes.

Term	Synonym	Structure
1. Autophagic vacuole	Cytolysome	Single or double membrane; contains morphologically recognizable cytoplasmic components, e.g. mitochondria
2. Cytosome	Primary lysosome "Virgin" lysosome	Almost any cytoplasmic organelle limited by a single unit membrane and of dubious identity
3. Multivesicular body		Single limiting membrane; contains inner vesicles similar morphologically to Golgi vesicles
4. Residual body	Telolysosomes "Post-lysosomes" Dense body	Membrane-lined inclusions probably of undigested residues appearing as whorls, myelin figures, membrane-fragments, etc.

able treatment of the particles and this was termed "breaking the latency". All of the treatments which were used to break the latency were known to affect the binding of lipid to protein and so could be expected to affect lipid-protein membranes. It appeared to de Duve and his colleagues that since all of the enzymes were hydrolytic enzymes, their containment in a sac might afford

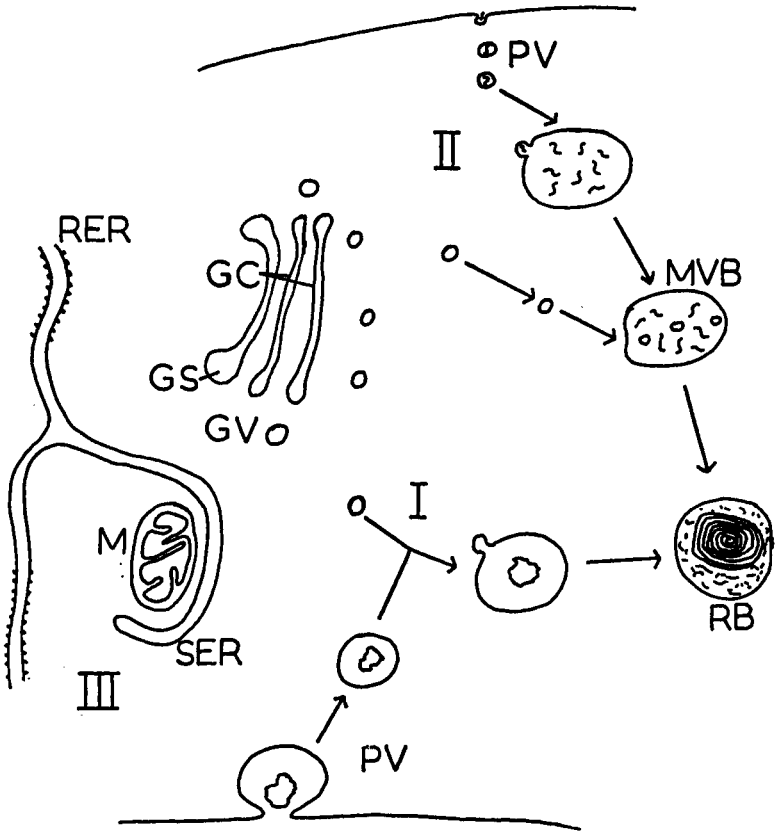

FIG. 1 I. Golgi vesicules (GV) or cytosomes are formed by Golgi saccules (GS) budding off Golgi cisternae (GC). These fuse with pinocytic vacuoles (PV) to form secondary lysosomes and then residual bodies (RB).

II. Alternatively, pinocytic vacuoles may fuse to form a larger vacuole which then incorporates cytosomes to form a multivesicular body (MVB). These can also give rise to residual bodies.

III. Autophagic vacuoles may arise by the enclosure of, for instance, a mitochondrion (M) within the smooth endoplasmic reticulum (SER), the whole then moving away. Enzymes within these vacuoles are thought to arise from the rough endoplasmic reticulum (RER) via the smooth endoplasmic reticulum. (After Novikoff *et al.*, 1964).

some protection to the cell itself and that the release of these enzymes into the cell might cause the death of the cell. He then coined the rather picturesque misnomer "suicide particles" to describe these light mitochondria or lysosomes (de Duve, 1959). Examination of isolated lysosomes in the electron microscope shows that they possess a single limiting membrane (and not the double membrane which is so typical of the cell in general) and have no detailed internal structure (Novikoff *et al.*, 1956). Optical and electron microscope cytochemical investigations on tissue sections of a wide range of animal tissues have also yielded confirmation of the presence of particles containing acid hydrolases (reviewed by Gahan, 1967).

Further studies at the electron microscope level in animal tissues have shown that "lysosomes" are not, in fact, a single series of particles each with a single limiting membrane as was originally thought but that there are a number of sub-cellular particles which contain hydrolase activities (Table II). These details have been unified to give a functional classification of lysosomes and lysosome-like particles in animal cells (Fig. 1).

II. Lysosomes in Plant Cells

A. BIOCHEMICAL STUDIES

In the early biochemical studies most plant biochemists attempted to apply directly the methods of analysis used by animal biochemists. The results of such experiments tended to discourage the majority of plant biochemists and a number of workers were unable to obtain any evidence for the presence of lysosomes. Of such experiments, few were published. However, Douglas *et al.* (1963) attempted to isolate lysosomes from tissues of cauliflower and bean. Rat liver was also used to allow assessment of the isolation procedure. They obtained a lysosomal fraction only from the rat liver and were completely unable to find anything resembling lysosome-like particles in the plant material. More encouraging were the results of Harrington and Altschul (1963) who attempted to isolate lysosomes from germinating onions. They obtained a light mitochondrial fraction which contained acid phosphatase activity, this activity being released into the supernatant fraction after treatment of the particles with Triton X-100. Little comment is needed to stress the differences in preparing sub-cellular fractions from plant tissues as opposed to preparing them from animal tissues. In consequence, the early negative biochemical results in attempts to identify lysosomes could have been due to the absence of such particles from plant tissues but could equally have been due to the need to modify quite severely the preparation techniques employed. Matile *et al.* (1965) used 48-hr-old seedlings of *Zea mays* or tobacco seedlings which were ground in the presence of sand in ice cold 7% sucrose buffered at pH 7·1. After filtration of the

homogenates through cheese cloth, they were centrifuged to remove sand, cell debris, nuclei and starch and the resulting cell-free extract was layered onto a linear density gradient ranging from 20% to 50% sucrose. The preparations were then centrifuged at 40,000 rev/min for 2½ hr (*Zea mays*) or 4½ hr (tobacco) and the contents of the tubes were collected in either 15 or 19 fractions, respectively. The fractions were analysed for acid protease, acid phosphatase, esterase and acid ribonuclease. In fraction 6 of the corn seedlings there were peaks of acid protease and esterase activity and part of the acid phosphatase activity. The relative density of the particles in this fraction was calculated to be 1·138 g cm^{-3} and they were comparatively rich in protein. There was a similar culmination of peaks of acid hydrolase activities in fraction 10 of the tobacco, corresponding to particulate material and appearing to be rich in protein. A second set of peaks of hydrolase activity for each of the seedlings occurred at about fraction 9 for corn and fraction 13 for tobacco. The density of the second peak for corn corresponded to approximately 1·105 g cm^{-3}. Again the second bands were also found to be rich in protein. In addition to the demonstration of banding of activities corresponding to certain groups of particles, it was also clear that portions of the total hydrolase activities were not attached to particles and upon centrifugation these enzymes remained among the soluble proteins of the cell-free extracts. Matile and his associates were not able to decide from these results whether the free enzyme molecules were normally free in the cell or whether they represented a fraction of the hydrolases which were originally bound to particles which had been somewhat damaged during the preparation procedure, especially since the relative amounts of free and particulate hydrolases varied considerably from one preparation to another. Semadeni (1967) has studied similar fractions and has found that acid hydrolases which include protease, phosphatase, non-specific esterase, ribonuclease, arylsulphatases A and B and amylase are also sedimentable from cell-free extracts of corn seedlings. After differential centrifugation of the extracts, the sedimentable hydrolysates recovered were mainly in the mitochondrial and microsomal fractions. It could be demonstrated that protease, phosphatase, ribonuclease and esterase of the mitochondrial fractions were contained in membrane-bound particles and isopicnic centrifugation of cell-free extracts in sucrose gradients revealed the presence of three particulate fractions carrying hydrolases. The heaviest of these had a relative density of 1·138 g cm^{-3} and contained the acid protease, acid phosphatase, acid ribonuclease and esterases. A lighter fraction with a relative density of 1·105 g cm^{-3} contained the same acid hydrolases. The specifically lightest cell fraction with a density of the order of 1·070 g cm^{-3} contained the acid hydrolases glucose-6-phosphatase, arylsulphatase C and small amounts of α- and β-amylase activities. This latter fraction also contained the NADH-diaphorase activity. β-Glucuroni-

dase, phospholipase C, lipase and arylsulphatases A and B, all of which are typical animal lysosomal enzymes, were completely absent from the cell-free extracts of corn seedlings. Matile (1966) also claims to have isolated vacuoles from the rootlets of corn seedlings, the vacuoles having densities higher than 1·029 g cm^{-3} and containing protease, acid ribonuclease, acid deoxyribonuclease, acid phosphatase and two different nonspecific esterases. The vacuoles from higher plant cells have been equated with organelles in which the processes of intracellular digestion can take place; in other words, they have been equated with the lysosomes of animal cells.

B. CYTOCHEMICAL STUDIES

Owing to the lack of positive biochemical data, some workers applied a cytochemical approach to assess if lysosomes were present in plant tissues. Jensen (1956) reported that acid phosphatase activity in root-tip cells was associated with particulate structures in the cytoplasm which he identified as mitochondria. These observations were later confirmed by Avers (1961) who found acid phosphatase activity at particulate sites in the root tips of *Phleum pratense* and *Panicum virginatum.*

Walek-Czernecka (1962) studied onion scale epidermal cells and showed that acid phosphatase and nonspecific esterase activities were associated with cytoplasmic particles. She further demonstrated (1965) that aryl-sulphatase, lipase, β-glucuronidase, β-galactosidase and acid deoxyribo-nuclease were also present at particulate sites in the cells of the onion scale epidermis.

Gorska-Brylass (1965) performed a similar series of studies but this time on pollen grains and pollen tubes of some 34 plant species and found acid phosphatase, β-glucuronidase, acid deoxyribonuclease and nonspecific esterase activities to be localized at particulate sites.

Gahan (1965) studied lysosome-like particles in plant tissues by attempting firstly to define the localization of the acid hydrolases in the cell and secondly to determine if the sites of localization responded in a manner expected of lysosomes as considered by the definition of the animal lysosomes. By using frozen sections of roots of *Vicia faba,* it was possible to prepare serial sections of tissue which appeared to be preserved in a manner approaching that of living tissue (Gahan *et al.*, 1967). Incubation of such cells for acid phosphatase activity using β-glycerophosphate as substrate showed that in the root meristem cells the hydrolase activity first appeared after 20 min of incubation and, furthermore, was at particulate sites in the cytoplasm (Gahan, 1965).

In order to test that the delay in observing a reaction was due to a true "latency" of the particles and not to the need to incubate the sections long enough to obtain a sufficient end-product of the enzyme reaction to be seen

with the light microscope, sections were pre-incubated in a pH 5 buffer alone for 10 min and then were transferred to the test medium. It was found that only 10 min of incubation was required in the test medium to obtain a reaction normally seen after the full 20 min incubation. As a further test of latency, sections were treated by freezing-and-thawing six times prior to incubation in the full reaction medium when it was found that only 2 min of incubation was required to demonstrate the presence of the particulate sites of activity which were normally seen after only 20 min of incubation in the untreated frozen sections. It would appear from these data that particles termed "lysosomes" by the animal biochemists could be demonstrated cytochemically. However, it was also required that more than one acid hydrolase should be present at these particulate sites if the fuller meaning of the term lysosome were to be possibly applied to plant cells. It was not possible to demonstrate arylsulphatase and β-glucuronidase in root cells of *V. faba* although β-glucuronidase was demonstrable in shoots of germinating pea seeds (P. B. Gahan and J. McLean, unpublished results). Esterase activity was demonstrable in roots of *V. faba* using either 5-bromo-indoxyl acetate (Holt, 1958) or naphthol AS D acetate (Burstone, 1962) as substrate. Moreover, it was shown (P. B. Gahan and J. McLean, unpublished results) that when acid phosphatase and esterase activities were present in the same cell, then these two hydrolases could be associated with the same particle (Fig. 2A, B, C).

If one observes the general distribution of acid hydrolases throughout a range of plant tissues at the light microscope level then, in addition to these sub-cellular particles or particulate sites, one can also observe that, when using β-glycerophosphate as substrate, acid phosphatase activity appears also in association with the chloroplasts and naphthol AS phosphatase with the plasmodesmata (J. McLean and P. B. Gahan, unpublished results). Furthermore, esterase activity in certain regions of the root-cap cells can be clearly seen in and between the cell walls (J. McLean and P. B. Gahan, unpublished results).

Electron microscope cytochemical studies have shown further that acid glycerophosphatase activity is present in the Golgi cisternae and that aryl-

FIG. 2 Frozen, longitudinal section through the stele of root of *V. faba*.
A. Naphthol AS phosphatase activity (blue) at particulate sites in the cells. Incubation for 30 min at 37°C in 0·05M-acetate buffer pH 5 containing naphthol AS Bl phosphate as substrate and Fast Dark Blue R as coupling agent.
B. Napthol AS esterase activity (red) at particulate sites in the cells. Incubation for 5 min at 37°C in 0·05M-acetate buffer pH 5 containing naphthol AS D acetate as substrate and Fast Red Violet LB as coupling agent.
C. Incubation for 5 min for esterase activity followed by 30 min incubation for phosphatase activity. The deposition of both red and blue end-product at the same site causes particles to appear purple, indicating the presence of the two enzymes at the same site. A, B and C × 320 (on diapositives).

sulphatase activity, as demonstrated with *p*-nitro-catechol sulphate, is present in the endoplasmic reticulum (Poux, 1967). Thus, while lysosome-like particles appear to be present from optical microscope studies, all localized reactions do not appear to resolve themselves as sub-cellular particles when viewed in the electron microscope.

III. Lysosomes and Spherosomes

In the early cytochemical studies of hydrolase activities in plant cells, Walek-Czernecka (1965) and Gorska-Brylass (1965) both attempted to determine the type of particle with which the hydrolase activity was associated, either by means of phase-contrast observation or by staining the particles with intra-vital dyes. The particles were readily differentiated from proplastids which did not appear black when observed with phase-contrast and were also readily distinguished from filamentous mitochondria, although some difficulty apparently was found in distinguishing between spherical mitochondria and these particles. However, if the preparations were stained with Janus green then the mitochondria took up the colour and appeared blue-green while the hydrolase-containing particles remained colourless. Similar studies by J. McLean and P. B. Gahan (unpublished results) have shown that either in tissue sections or in cell-cultures from crown gall tumours it was possible to obtain a Janus green reaction at mitochondrial sites and an esterase activity associated with a second group of highly refractile particles in the same cell.

Observations on particles of diameter 0·3–0·8μ in pollen tubes showed that they had peculiar optical properties and somewhat variable shapes; they appeared to be present as ". . . swollen rings, or sickles, or crescents, or oglomerated nubs inside a delicate capsule" whereby the density varied (Gorska-Brylass, 1965). The particles were also distinguished from simple fat droplets by means of cytochemical reactions. Moreover, in the growing pollen tubes, the fat droplets tended to coalesce at frequent intervals to form larger drops while the hydrolase-containing particles tended to remain apart.

The general characteristics of these hydrolase-containing particles, as described by Walek-Czernecka (1962), Olszewska *et al.* (1965) and Gorska-Brylass (1965), corresponded closely to the previously recorded description of the spherosomes in plant cells (Dangeard, 1919; Sorokin and Sorokin, 1966). Moreover, the two hydrolase-containing fractions from corn seedlings isolated by Semadeni (1966) also gave responses characteristic of spherosomes when studied with fluorochromes.

Spherosomes have been characterized in plant cells by electron microscope studies (Frey-Wyssling *et al.*, 1963; Mühlethaler, 1955; Perner, 1957; Hohl, 1960; Paleg and Hyde, 1964; Sorokin and Sorokin, 1966). Electron microscope

observations on the hydrolase-containing particles isolated from corn seedlings (as well as on the intact seeds and seedlings) by Matile *et al.* (1965) showed the particles to correspond to spherosomes which, in general, appear as an electron-dense stroma surrounded by a single membrane.

Very little is known of the chemical composition of spherosomes due to the difficulties encountered in isolating them from plant tissues. Cytochemical methods have revealed them to react freely with lipid stains (Drawert and Mix, 1962; Ziegler, 1953) as well as with phospholipid indicators (Sorokin and Sorokin, 1966) and with specific reagents for proteins (Perner, 1952; Jarosch, 1961). From the nature of the enzyme cytochemical studies it is possible that at least part of this protein is enzymic. Sorokin and Sorokin (1966) were unable to identify carbohydrate material by the PAS reaction.

IV. Formation and Function of Spherosomes

Electron microscope studies of spherosomes do not show such a wide range of particles containing hydrolases as are found in animal cells and it would be difficult initially to form an equivalent intra-cellular digestion pathway to that occurring in animal cells. Frey-Wyssling *et al.* (1963) have suggested that the spherosomes form by budding from the endoplasmic reticulum, much in the same way as some workers have considered for primary lysosomes (Brandes, 1965). However, there is no clear-cut evidence for the occurrence of pinocytosis within cells of the intact plant organ and, until contrary evidence is produced, it is difficult to envisage an identical intra-cellular digestive system operating in plant cells.

Biochemical and cytochemical studies clearly indicate the formation of lipid droplets within spherosomes and it appears that on ageing, spherosomes become transformed into lipid droplets (Dangeard, 1919; Frey-Wyssling *et al.*, 1963). What is not evident is whether the contents of the original spherosome are transformed into lipids or whether lipid synthesized elsewhere is accumulated within the spherosomes or whether the spherosomes are themselves the sites of lipid synthesis. It is known from biochemical studies that fatty acid synthesis can occur in mitochondrial fractions of plant tissues (reviewed by Mead, 1963). Thus, the third suggestion seems initially the most interesting especially since Semadeni (1966) was also able to demonstrate the incorporation of ^{3}H-acetate into lipids of isolated spherosomes from which he concluded that lipid synthesizing enzymes were present on the spherosomes.

This function alone, however, does not completely account for the presence of acid hydrolases in spherosomes and further studies are required to elucidate this point.

V. CONCLUSIONS

From the foregoing studies, it would appear that there are gross differences between the hydrolase-containing particles in plant and animal cells. Further differences can be seen in a study of the mitotic cycle. Robbins and Marcus (1963), Allison (1965) and Maggi (1966) have presented evidence that during mitosis in animal cells, there is an increased permeability of the lysosomal membrane. Similar studies on plant cells have shown that there is no such behaviour in mitotic cells from root and shoot meristems of *V. faba* or shoot meristems of *Solanum lycopersicum* (P. B. Gahan and J. McLean, unpublished results). The hydrolase-containing particles behave similarly in both mitotic and interphase cells. In mitotic cells in differentiation regions, however, there is a much higher esterase activity which appears to be correlated to differentiation rather than with division.

Since there are such marked differences between the hydrolase-containing particles of plant and animal cells, it would seem that the term "lysosome" should be reserved for the particles present in animal cells and "spherosome" for the particles present in plant cells.

ACKNOWLEDGEMENTS

Financial assistance by the Science Research Council for support of some of the work described in this article is gratefully acknowledged.

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