

Function of Lysosomes and Lysosomal Enzymes in the Senescing Corolla of the Morning Glory (*Ipomoea purpurea*)

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Received 12 November 1970

ABSTRACT

The rapid senescence of the *Ipomoea* corolla is characterized by the breakdown of protein and nucleic acids. At the onset of wilting the activities of deoxyribonuclease (DNase), ribonuclease (RNase), and β -glucosidase are increased dramatically, while other hydrolytic activities such as the actions of protease, aminopeptidase, α -glucosidase, phosphatase, esterase, and α -amylase are only slightly changed.

Isolated corolla discs show a course of senescence similar to that of the intact organ. When floating on solutions of cycloheximide the activities of DNase, RNase, and β -glucosidase do not increase. Actinomycin D inhibits the increase in RNase activity. It is concluded that protein synthesis is a prerequisite for the changes in these enzyme activities in the senescing corolla.

The function of the lysosomal compartment in the process of senescence is illustrated by electron micrographs showing the autophagic activity of vacuoles. The last phase of senescence is characterized by the breakdown of the tonoplast and complete digestion of the cytoplasmic constituents in the autolysing cells.

INTRODUCTION

Schumacher (1932) has observed a rapid decrease of protein nitrogen in flowering and wilting petals of various species. Fast-wilting corollas, such as those of bindweeds, lose the bulk of their nitrogen in a short time period. This aspect of senescence implies the occurrence of lytic processes taking place in the senescing organ. Recent experiments with plant tissues have shown that synthesis and breakdown of nucleic acids and protein may occur simultaneously (Sampson, Katoh, Hotta, and Stern, 1963; Bidwell, Barr, and Steward, 1964). Moreover, synthesis of protein (Hall and Cocking, 1966; Simon, 1967; Atkin and Srivastava, 1970) and RNA (Ecklund and Moore, 1969) has been demonstrated in senescing plant organs in which the levels of total protein and RNA decline. The compartmentation of synthetic and lytic processes in the cell (the latter taking place in lysosomes) seems to represent an organizational prerequisite of turnover of these compounds. From this viewpoint the changes in total protein and nucleic acids appear as the resultants of changing rates of synthesis and digestion.

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The present study deals with an attempt to correlate lysosomal enzyme activities with the breakdown of protoplasmic material in the wilting corolla of the bindweed *Ipomoea*. Experiments were done to examine the changes of hydrolase activities in the course of senescence.

Recent attempts to isolate plant vacuoles have yielded considerable evidence favouring the lysosomal nature of these organelles (Matile, 1966, 1968a). In the present study the possible function of the lysosomal compartment in senescence and autolysis has been investigated by means of electron microscopy.

MATERIAL AND METHODS

Object and culture

Ipomoea purpurea (var. *rubro coerulea praecox*) were sown in neutral garden soil and cultivated in the greenhouse or outdoors. During winter a photoperiod of 14 h was maintained artificially.

Collection, homogenization, and extraction of corollas

At each flowering stage 15 flowers, equal in size and appearance, were picked and cooled quickly to 0 °C. In some cases funnels were used instead of whole corollas.

Extraction for estimating carbohydrates. Five corollas from each sample were immediately chopped and killed in hot 80 per cent ethanol. Sugars were extracted with the same solvent and starch was hydrolyzed with 52 per cent perchloric acid (Glegg, 1956).

Extraction for estimating enzyme activities. Ten chopped corollas were homogenized (Sorvall Omnimixer, step 7, 3 min) with 10 ml of carbonate buffer (0.025 M, pH 6.5 0 °C) and then filtered through mesh bolting silk (75–100 μ m). The residue was ground together with 3 g quartz sand (0.1–0.8 mm diameter) and 5 ml of carbonate buffer with a mortar and pestle and centrifuged (1 min, 500 g; 10 min, 2000 g). Supernatants and the filtrate were pooled and adjusted to a final volume of 25 ml. This extract was used for the estimation of enzyme activities and substances. Its pH varied between 6.5 and 6.8.

Estimation of protein and nucleic acids. Aliquots of the above extracts were precipitated twice with cold perchloric acid (0.2 N final concentration); the pooled supernatants were the acid-soluble fraction. The sediment was washed twice with 0.2 N HClO₄ and extracted with chloroform-methanol (2:1) (v/v) to remove the lipids. The nucleic acids were hydrolyzed in 0.5 N HClO₄ (20 min, 90 °C). The proteins were extracted with 1 N NaOH at room temperature. The yield in proteins was higher if the extraction of lipids was omitted.

For the estimation of deoxyribonucleic acid (DNA), five corollas were ground twice with a mortar and pestle together with 3–10 ml 0.2 N HClO₄ and centrifuged (10 min, 2000 g). The sediment was washed twice with 0.2 N HClO₄ and DNA was hydrolyzed with 1.6 N HClO₄ at 37 °C for 40–8 h (Wollgiehn, 1961).

Determinations

Ribonucleic acid (RNA) was estimated with orcinol (Mejbaum, 1939) and DNA with diphenylamine (Burton, 1956). The method of Lowry, Rosebrough, Farr, and Randall (1951) was used for the estimation of protein. The anthrone method (Glegg, 1956) was used for the determination of starch.

Estimation of hydrolase activities

Incubations of 0.1 ml of extract from corollas with the substrates listed below were performed at 37 °C.

(a) *Natural substrates. Nucleases.* Native thymus DNA or acid denatured yeast-RNA (0.4 ml; 0.2 per cent in 0.1 M acetate buffer pH 5.25). The activities were determined according to Ambellon and Hollander (1966). The pH ranges for optimal activities are 5.0–5.7 (DNase) and 4.5–5.5 (RNase).

Protease. Acid denatured haemoglobin (0.3 ml; 1 per cent in 0.1 M acetate buffer pH 4.2). The reaction was stopped by adding 0.2 ml 1 N HClO₄ and the acid-soluble fission products were estimated with the Folin phenol method (Greenberg, 1961, modified by Matile, 1964).

α-amylase. The substrate was 0.7 ml of 0.03 per cent starch (Analar) in 0.07 M phosphate buffer pH 6.0. The decrease of the iodine-starch-complex was estimated in a spectrophotometer at 590 nm (Street, 1956).

(b) *Synthetic substrates.* Enzyme aliquots were incubated together with 0.2 ml of substrate dissolved in buffer and the reaction was stopped with 0.2 ml 1 N HClO₄. After addition of 2.5 ml 0.2 M sodium carbonate the reaction product was estimated by its optical density at 400 nm.

α-glucosidase. 2 mg/ml of *p*-nitrophenyl- α -D-glucopyranoside in 0.1 M citrate buffer pH 5.7.

β-glucosidase. 1 mg/ml of *p*-nitrophenyl- β -D-glucopyranoside in 0.1 M acetate buffer pH 5.0.

Phosphatase. 2 mg/ml *p*-nitrophenylphosphate in 0.1 M citrate buffer pH 5.7.

Acetylase. 5 mg *p*-nitrophenylacetate dissolved in 1 ml methanol (pH 1 with HCl) at 0 °C and diluted with 4 ml 0.1 M citrate buffer pH 6.2.

Leucylaminopeptidase. 0.75 mg/ml L-leucyl-4-nitroanilide dissolved in 0.05 M carbonate buffer pH 9.5.

Experiments with isolated corolla discs

Corolla discs (1 cm diameter) were punched from the peripheral region of the funnel with a corkborer. Samples of 20 discs were floated (upper epidermis facing the solution) either on distilled water or on 12 μ g/ml cycloheximide in covered Petri-dishes and incubated at room temperature and in daylight. Discs from one flower were equally distributed among the samples. At intervals the incubated discs were ground by mortar and pestle together with 2 ml of 0.05 M tris (hydroxymethyl) aminomethane buffer pH 7.5 and the homogenate filtered through a paper filter. The filtrate was used for estimating hydrolase activities.

Electron microscopy

Pieces of corolla 2 × 2 mm were fixed (1.5 per cent glutaraldehyde, 0.4 per cent formalin, 0.025 M cacodylate buffer pH 7.2, 0 °C) for 15 h. Osmium tetroxide (1 per cent) was used for post-fixation. Sucrose was added to both fixatives to give a total molarity of 0.33 M. After contrasting with 2 per cent uranyl acetate the tissue was dehydrated in ethanol and acetone, and embedded in Epon resin. The thin sections were contrasted by the method of Reynolds (1963) and examined in a Siemens Elmiskop I electron microscope.

RESULTS

The corolla of *Ipomoea* comprises a tube which opens out to form a funnel. Usually the flower opens at 5–6 a.m. and remains open for 6–11 h. Upon wilting the corolla rolls up its funnel from the edge. After about 6 h the funnel is rolled up; wilting then proceeds to the tube of the corolla, and after 1–2 days the corolla is abscised (Plate 1).

Activity of hydrolytic enzymes and content of their potential protoplasmic substrates

In the *Ipomoea* corolla the wilting period is characterized by the rapid breakdown of nucleic acids and protein. It is remarkable that the RNA content is already decreasing in the late elongation period, while the DNA and the protein content decrease mainly during the wilting period. In Fig. 1A and B we have compared the changes in the content of macromolecular compounds and in the activities of some hydrolases. The breakdown of nucleic acids is reflected by a rapid increase in the activity of the nucleases. It is likely that the two nucleases are substrate-specific enzymes, since the activity of an unspecific phosphodiesterase (substrate:

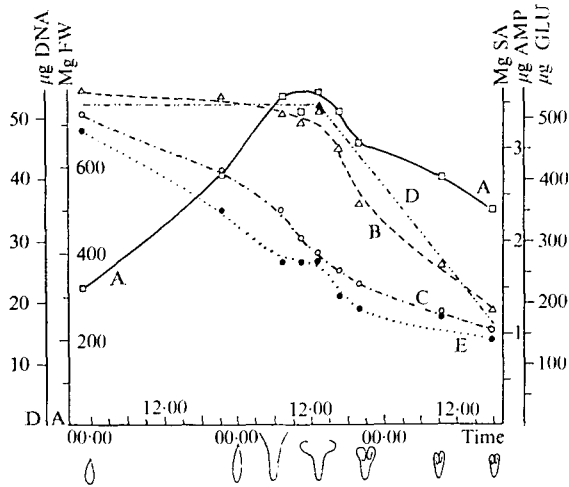


FIG. 1A. Content in (A \square — \square) freshweight, (B Δ — Δ) protein (mg serumalbumin), (C \circ — \circ) RNA (μg AMP), and (E \bullet — \bullet) starch (μg glucose) per corolla. Content in (D) DNA (μg DNA) per corolla from another experiment.

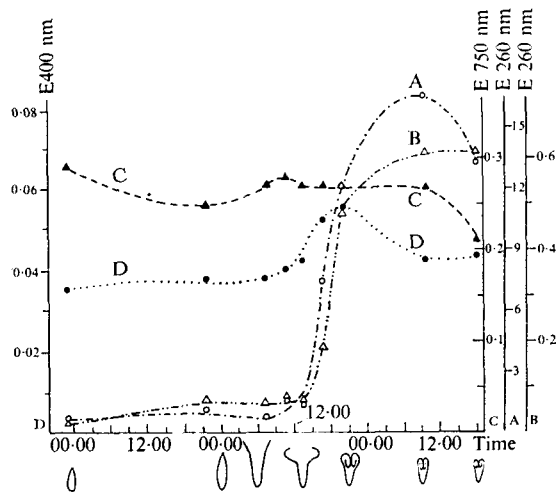


FIG. 1B. Relative activities of acid hydrolases per corolla: (A \circ — \circ) RNase, (B Δ — Δ) DNase, (C \blacktriangle — \blacktriangle) protease, and (D \bullet — \bullet) α -glucosidase.

The sketches below time axis indicate the shape of corolla.

bis-(*p*-nitrophenyl)-phosphate, pH 5.5) does not change significantly during the wilting period; in addition RNase is over 20 times more active than DNase.

A comparison between DNA content and DNase activity in the senescing tissue shows that the increasing enzyme activity is accompanied by a rapid breakdown of its substrate. In contrast, the protease activity changes only slightly while the protein content decreases rapidly (Fig. 1A, B). One has to conclude from this that the breakdown of macromolecules is not exclusively the concern of digestive enzymes. Considering the compartmentation of lytic and synthetic processes, the

decreasing protein content in the senescing tissue could indicate that the bulk of protein comes into contact with the existing proteases (Fig. 1A, B).

The starch content decreases steadily; α -glucosidase activity increases only temporarily at the onset of wilting, while the α -amylase activity increases by about 30 per cent during the flowering period and thereafter remains constant (Figs. 1A and 2).

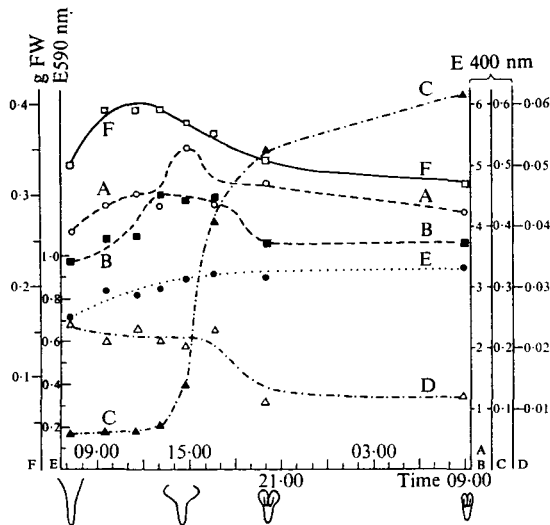


FIG. 2. Relative activities of hydrolases per funnel of corolla: (A \circ — \circ) phosphatase, (B \blacksquare — \blacksquare) esterase, (C \blacktriangle — \blacktriangle) β -glucosidase, (D \triangle — \triangle) aminopeptidase, and (E \bullet — \bullet) α -amylase. (F \square — \square) freshweight per funnel of corolla.

Acid phosphatase and esterase are the most active hydrolases in corolla funnels reaching their maximal activities at the beginning of wilting (Fig. 2). β -Glucosidase activity increases tenfold during the wilting period but is about 10 times less active than the acid esterase. The alkaline leucylaminopeptidase loses half of its activity during the wilting period and is about 200 times less active than the acid esterase.

Protein synthesis in isolated discs of corollas

In isolated discs of newly expanded corollas the RNase activity increases many-fold within a few hours. As compared with the intact organ the isolated discs behave similarly with regard to RNase, whereas the commencement of the increase of DNase and β -glucosidase activities is slightly retarded (Fig. 3).

The increase of RNase is strongly inhibited by antibiotics known to interfere with protein synthesis. As demonstrated by the results listed in Table 1, cycloheximide and actinomycin D prevent or diminish the increase of this enzyme activity in isolated discs. The effect of actinomycin D depends on its concentration and also on the form and size of the sections. It is most likely that diffusion of this inhibitor to the site of its action in the nuclei is slow. Cycloheximide also completely prevents the increase of DNase and β -glucosidase activity (Fig. 3). Therefore,

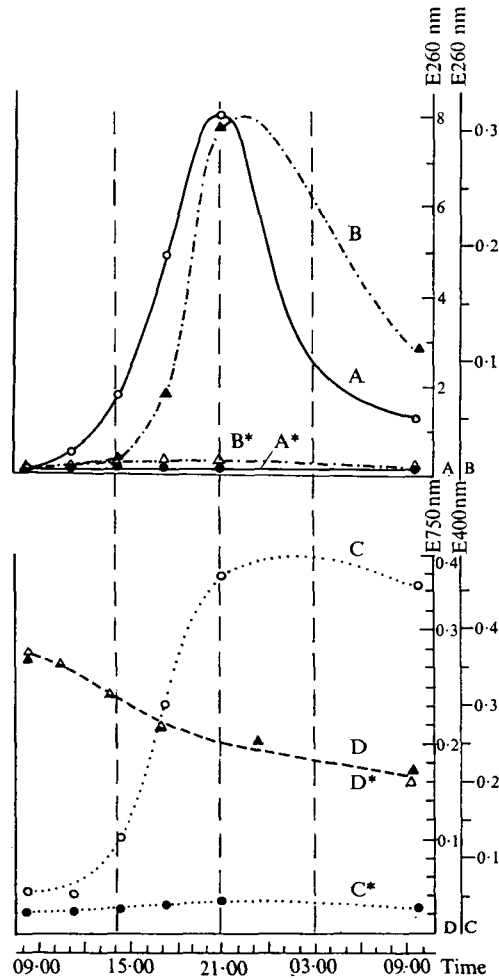


FIG. 3. Discs from newly expanded funnels of corolla floating on distilled water or on solution of cycloheximide (*). Start of experiment at 8.20 a.m. Relative activities of RNase (A, A*), DNase (B, B*), β -glucosidase (C, C*), and relative protein content (D, D*) per disc.

TABLE 1. Change of RNase activity in isolated corolla sections of different shape and size floating on water, on solutions of cycloheximide and solutions of actinomycin D. RNase activity is related to the activity after 7.5 h in discs floating on water (100 per cent)

	Start	After 7.5 h				
		0	20	50	100	200
(a) Cycloheximide $\mu\text{g/ml}$	0	0	12			
Circular sections $d = 10 \text{ mm}$	5%	100%	5%			
(b) Actinomycin D $\mu\text{g/ml}$	0	0	20	50	100	200
Circular sections $d = 10 \text{ mm}$	5%	100%	89%	77%	—	—
Rectangular sections $2 \times 10 \text{ mm}$	5%	100%	74%	57%	—	—
Square sections $3 \times 3 \text{ mm}$	4%	100%	—	67%	51%	26%

protein synthesis seems to be a prerequisite for the increase of these enzyme activities in the senescing corolla.

In senescing discs cycloheximide has a negligible effect on the change in total protein (Fig. 3). Consequently the possible *de novo* synthesis of the above enzymes seems to have no effect on the over-all protein content.

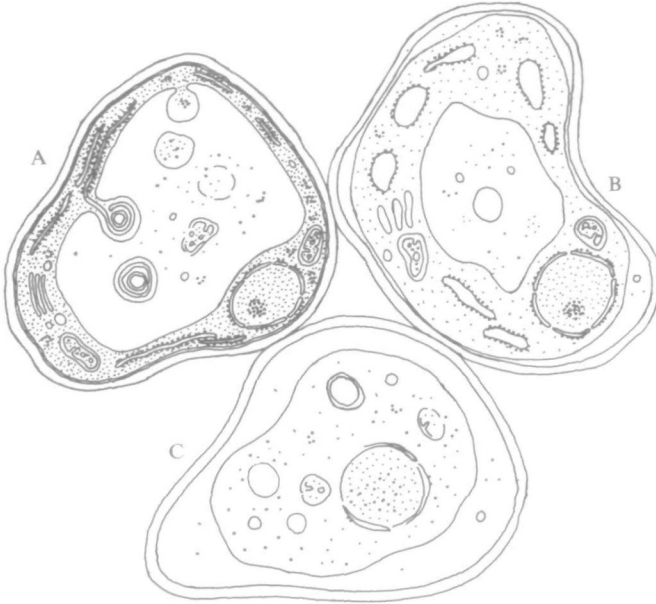


FIG. 4. Morphological changes in mesophyll cells of the senescing *Ipomoea* corolla.

A. Autophagic activity of the vacuole; invaginations of the tonoplast result in the sequestration of cytoplasmic material into the lysosomal compartment.

B. Shrinkage of the vacuole, dilution of the cytoplasm and inflation of cytoplasmic membrane systems.

C. Autolysis is initiated by the breakdown of the tonoplast.

Fine structure of the senescing corolla

In the course of senescence the cytological changes occur asynchronously not only in the different tissues of the corolla but also over the whole organ in which ageing proceeds from the periphery of the funnel towards the tube. Plate 2A gives an example of this heterogeneity. Nevertheless, the sequence of changes is similar in all the cells. A reconstruction of the changes observed is compiled in the scheme of Fig. 4.

Autophagic activity of the large central vacuole can be observed in practically all of the cells and in any tissue. It is indicated by the presence of cytoplasmic material in the cell sap. Frequently decaying mitochondria, ribosomes, and membranes can be observed in the digestive compartment (Plate 2). This material seems to be transported into the vacuole by invaginations of the tonoplast which later are pinched off. The resulting intravacuolar vesicles decay and the cytoplasmic material released becomes exposed to the lysosomal enzymes. The invagination of the tonoplast is often connected with the formation of membrane whorls originating from the ER (Plate 2c). In certain cells autophagic activity may

continue until only a very thin layer of dense cytoplasm is left behind. In extreme cases the plasmalemma is locally adjacent to the tonoplast. At this stage of development the complete set of organelles having a normal structure, mitochondria, rough ER, Golgi producing vesicles, proplastids, and nuclei can still be observed.

This appearance of the cytoplasm changes as the cells approach autolysis. The volume of the cell sap decreases, the vacuoles shrink, and the cytoplasm assumes a dilute appearance (Plate 3A). The loss of turgor is probably connected with the rapid export of sugars at the onset of wilting (Winkenbach and Matile, 1970). It can frequently be observed that the dilution of the cytoplasm is preceded by the shrinkage of the whole protoplast. It seems, therefore, that the plasmalemma gradually loses its semi-permeability. This change may in turn be responsible for the increasing volume of free space observed by Sacher (1967) in senescing plant tissues. The resulting dilution of the cytoplasm in addition involves the inflation of the cytoplasmic membrane systems; the ER cisternae (Plate 3B) and also mitochondria and dictyosomes swell considerably. Whether the cells are still metabolically active at this stage is uncertain. If the disorganization of the protoplast is the morphological criterion of death, the cells die when the continuity of the tonoplast is abolished (Plate 3C). This event is followed by the mixing of the cytoplasmic constituents with the cell sap and results in the autolytic degradation of the whole protoplast (Plate 3D, E). Mitochondria, ER cisternae, and nuclei can be observed at various degrees of degradation. At the end of this process the cells appear to be completely empty.

It is unlikely that the above observations represent artifacts of fixation. At intermediate stages of senescence the whole sequence of events can frequently be observed in one section.

A detailed analysis of specimens taken from the periphery of the corolla fixed at different points in time has yielded the following behaviour of the individual tissues. The mesophyll is the first tissue in which autolysis can be observed. Sporadically autolysing mesophyll cells are present in the mature bud and during the early stages of flowering. Most of them begin to autolyse when the funnel rolls up. After completion of this process only a few mesophyll cells are still intact, and the whole tissue is completely devoid of cytoplasm in the morning after the day of flowering. In contrast the epidermal cells show only autophagic activity until the funnel is rolled up completely. In this tissue autolysis commences in the late evening of the day of flowering and is not completed by the morning of the following day. The vascular bundles show a more complicated behaviour. Parenchyma cells behave in a way similar to that described above for the mesophyll, whereas intact sieve tubes and companion cells can be observed throughout the senescence of the corollas.

DISCUSSION

Function of hydrolytic enzymes in the senescing corolla

The most striking biochemical event taking place in the senescing corolla concerns the 40–50-fold increase of the nuclease activities. Similar increases in RNase activity have also been observed in detached leaves of tobacco (Balz, 1966), oats

(Udvardy, Farkas, and Marrè, 1969), and other senescing plant tissues (Hanson Wilson, Crispeels, Krueger, and Swanson, 1965; Rhodes and Woollorton, 1967). In detached barley leaves the activities of RNase and DNase rise considerably during senescence (Srivastava, 1968). Ageing tomato leaves have a higher activity than young ones (McHale and Dove, 1968). Thus, the increase in nuclease activity seems to be a common feature in senescing plant organs, though some authors have reported declining RNase activities in ageing tissues (see Phillips and Fletcher, 1969).

Although antibiotics known to interfere with protein synthesis inhibit the increase of RNase, DNase, and β -glucosidase activity in isolated discs of *Ipomoea* corollas it is not certain that the respective enzyme proteins are formed *de novo*. However, protein synthesis is a prerequisite of the phenomenon and senescence appears to involve the production of certain proteins during a period of rapidly decreasing total protein content.

While there is no clear correlation between RNase activity and RNA content, the remarkable increase of DNase is followed by a considerable breakdown of DNA. Therefore, the intracellular localization of these hydrolases and their potential substrate seems to represent a factor which controls the breakdown process. The function of the nucleases in senescence appears from the finding that about 80 per cent of the nucleic acids present in the mature corolla are broken down during the first 24 h of wilting. Concomitantly the content of soluble phosphates is reduced by 75 per cent (Winkenbach, 1970) suggesting that the products of hydrolysis are withdrawn from the corollas.

In contrast to the nucleases the proteolytic activity in senescing corollas and in other plant organs changes only little. Balz (1966) and Kawashima, Fukushima, and Tamaki (1967) have shown this in detached tobacco leaves; Anderson and Rowan (1965), using discs from young, old, and senescing leaves of the same species, obtained similar results. Nevertheless, during senescence the protein level decreases markedly in tobacco leaves as well as in *Ipomoea* corollas. Therefore, at onset of senescence portions of protein seem to become exposed to already existing proteases. The electron micrographs of senescing *Ipomoea* corollas suggest that this process involves either the autophagic activity of the vacuole or the rupture of this lysosomal compartment.

The function and the natural substrate of β -glucosidase, an enzyme showing a conspicuous increase in activity in the senescing *Ipomoea* corolla are not known. Possible substrates are flavonoid compounds since most of them are β -glucosides (Harborne, 1964). In fact, a β -glucosidase from *Impatiens* petals hydrolysing both *p*-nitrophenyl- β -glucopyranoside and pelargonidin-3-monoglucoside has recently been partially purified (Mansell, 1969). Thus the corresponding enzyme of *Ipomoea* could be responsible for the breakdown of the anthocyanin taking place upon wilting.

Function of the lysosomal compartment in the senescing cells

The interpretation of the electron micrographs is based on the assumption that the vacuoles represent the lysosomal cell compartments. Although attempts to

isolate vacuoles from protoplasts prepared from the *Ipomoea* corolla have not been successful, and therefore a direct proof of the lysosomal nature of this organelle cannot be presented, the above assumption nevertheless seems to be justified. Vacuoles isolated from fungal and higher plant cells contain digestive enzymes in a concentrated form and, in addition, cytochemical work has shown that the lysosomal marker-enzyme, acid phosphatase, is localized in this organelle (reviews by Matile, 1968*b*, 1969).

The lysosomal nature of plant vacuoles furthermore appears from the presence of cytoplasmic material which several authors have observed at various stages of degradation (Poux, 1963; Sievers, 1966; Thornton, 1968; Hébaut, 1969; Bowes, 1969). In meristematic vacuoles of root tips the autophagic activity involves an activity of the tonoplast similar to that observed in the *Ipomoea* corolla: the vacuolar membrane invaginates and forms intravacuolar vesicles containing cytoplasmic material; these vesicles seem to decay and their contents are subsequently digested by the attack of the lysosomal enzymes (Matile and Moor, 1968). This mechanism, has also been observed by Coulomb and Buvat (1968) and Wardrop (1968), and the formation of myelin-like membrane whorls seems to be a conspicuous feature of autophagy in plant cells (see Bowes, 1969).

A comparison of the biochemical and morphological changes during wilting of the *Ipomoea* corolla shows that the initial decrease in RNA and protein, which has already commenced when the flower bud reaches maturity, must be due to autophagic activity. Since the rapid increase in nuclease activity takes place during the period in which the electron micrographs indicate an increasing number of autolysing cells, it is likely that this process immediately precedes autolysis. It requires protein synthesis and must therefore take place in living cells. An increase of respiratory activity observed during the first period of wilting (Winkenbach, 1970) could reflect the energy requirement of protein synthesis. Autolysis would then be responsible for the rapidly decreasing nucleic acid and protein content during wilting. Morphologically autolysis seems to be initiated by the breakdown of the tonoplast. Although the disappearance of this membrane has been recognized as a conspicuous feature of senescent plant cells (Shaw and Manocha, 1965; Butler, 1967), it has not been correlated with the breakdown of chloroplasts, mitochondria, and nuclei. Considering the lysosomal nature of vacuoles it is reasonable to assume that the abolition of compartmentation of digestive enzymes results in cell death.

Autolysis seems to follow a distinct pattern which guarantees an organized withdrawal of the products of autolysis: the phloem of the vascular bundles seems to remain intact as long as breakdown products are exported, presumably into the developing flower buds and into the seeds.

ACKNOWLEDGEMENTS

The authors are indebted to Miss R. Rickenbacher and Mr. P. Frick for valuable technical assistance and to the Swiss National Science Foundation for partial financial support.

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EXPLANATIONS OF PLATES

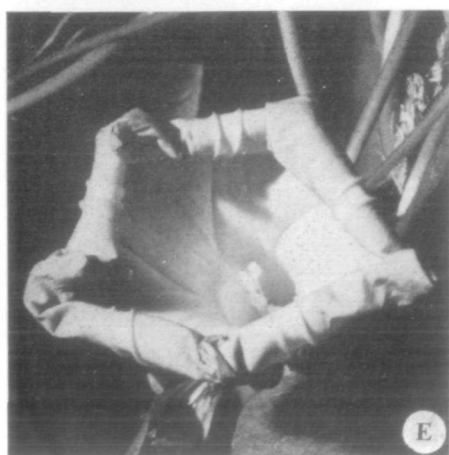
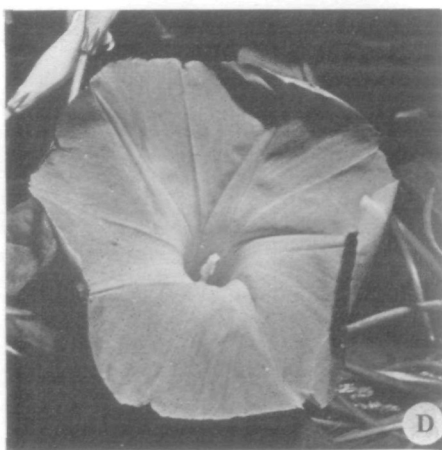
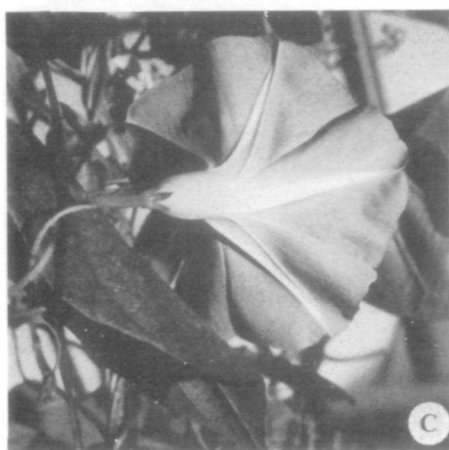
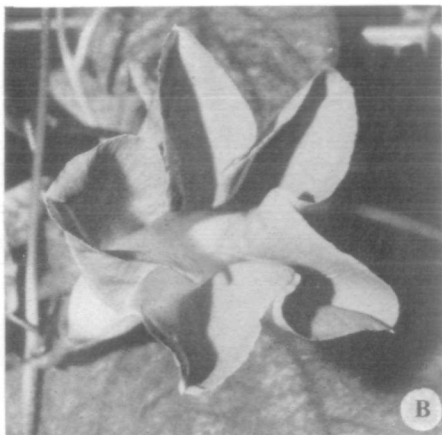
PLATE 1

Development of the *Ipomoea* corolla.

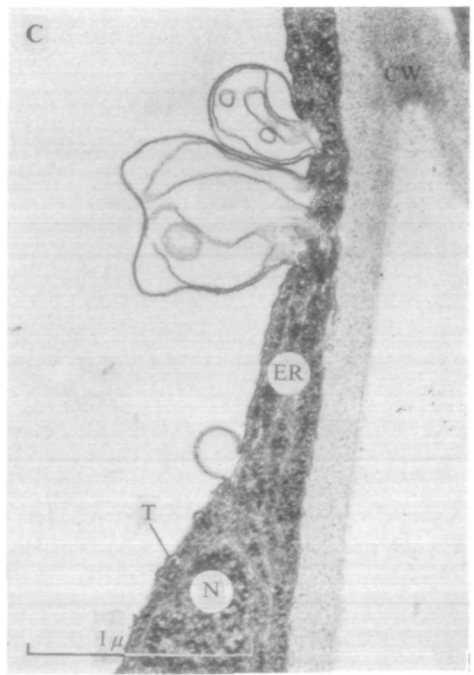
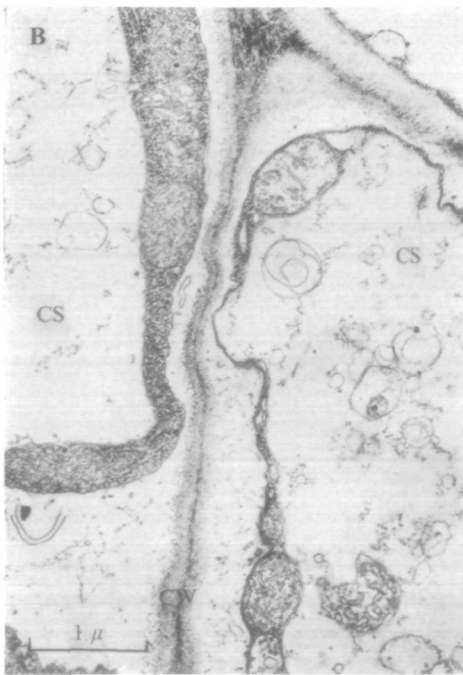
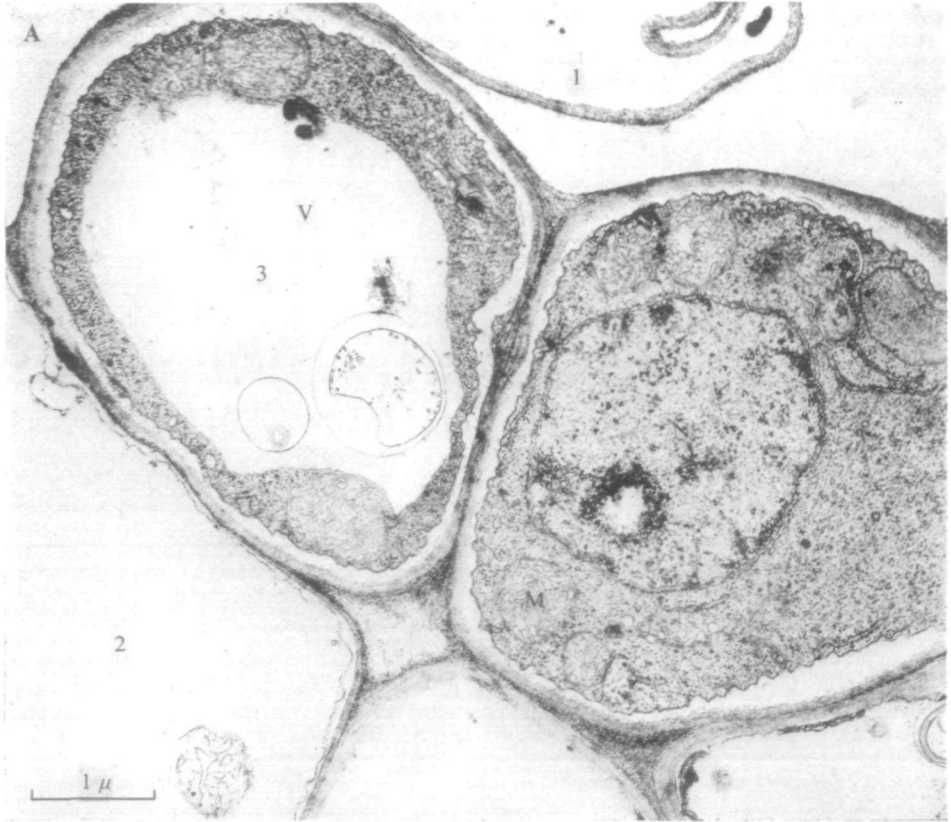
- A. Mature flower bud in the early morning of the flowering day. B. Expanded funnel at 6 a.m. C. Ribs whose rigidity is responsible for the shape of the funnel. D. First signs of wilting in the early afternoon. E. Partially rolled up funnel at 5 p.m. F. Corolla in the morning after day of flowering.

Key for Plates 2 and 3

N nucleus	ER endoplasmic reticulum
M mitochondrion	R ribosome
V vacuole	PL plasmalemma
T tonoplast	CW cell wall
CS cell sap	



PH. MATILE AND F. WINKENBACH—PLATE 1



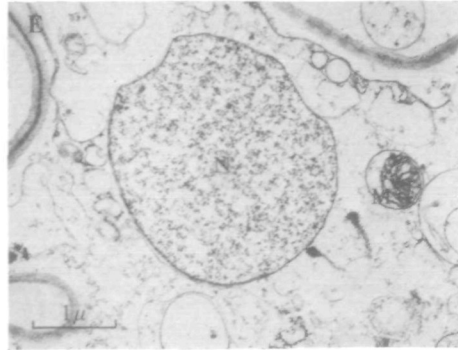
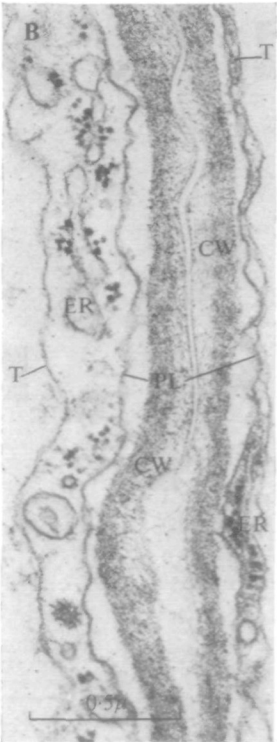
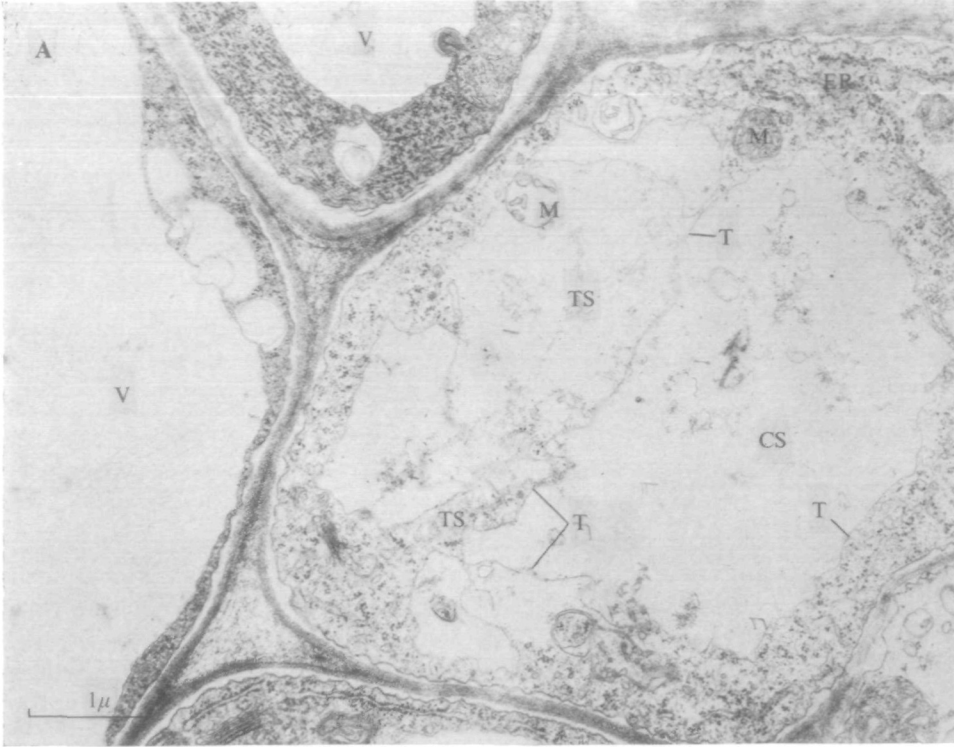


PLATE 2

- A. Edge of vascular bundle showing cells at different stages of senescence. The mesophyll cell (1) is completely empty and autolysis is nearly completed in cell 2. Autophagic activity is conspicuous in cell 3.
- B. Autophagic activity in cells of a vascular bundle at early wilting. Note the decaying mitochondrion, ribosomes, and membranes in the cell sap.
- C. Invaginations of the tonoplast containing ER membranes.

PLATE 3

- A. Shrinkage of the vacuole and dilution of the cytoplasm in a senescing cell of a vascular bundle. ts: transvacuolar strands.
- B. Adjacent mesophyll cells showing ER strand with normal (right) and inflated appearance (left).
- C. Breakdown of the tonoplast.
- D. Autolytic breakdown of cytoplasmic organelles of a mesophyll cell after mixing of cytoplasm and cell sap.
- E. Partially degraded nucleus in an autolysing mesophyll cell.