groupements hydroxyle en para et méta (acide caféique) soit deux groupements méthoxy en méta, encadrant un groupement hydroxyle en para (acide sinapique) atténue profondément l'effet inhibiteur observé avec l'acide férulique. Enfin, l'acide chlorogénique étant plus inhibiteur que l'acide caféique, on peut penser que la nature de la chaine latérale intervient. Le composé le plus actif est l'acide férulique, possédant en para et en méta respectivement les groupements hydroxyle et méthoxy.

Seule de toute la deuxième série de composés, la coumarine affecte fortement, aux concentrations élevées, l'élongation de la première feuille de blé. C'est le seul produit dépourvu de substituant.

Nous pensons, grace à ces quelques exemples, avoir pu montrer l'intérêt d'utiliser un matériel végétal tel que la première feuille de blé pour l'étude d'interactions impliquant GA_3.

Nous tenons à remercier vivement Mademoiselle Bulard, Professeur, pour l'aide bienveillante et les conseils éclairés qu'elle nous a prodigués au long de ce travail ainsi que le Dr. B. V. Milborrow pour l'échantillon d'(±)-acide abscissique qu'il a généreusement mis à notre disposition.

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A Cytochemical Study of the Leaf-Gland Enzymes of Insectivorous Plants of the Genus *Pinguicula*

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Summary. Cytochemical methods have been used to study the distribution of acid phosphatase, esterase, ribonuclease, amylase and protease activity in the stimulated and unstimulated leaf glands of *Pinguicula grandiflora*, *P. vulgaris*, *P. lusitanica*, and *P. caudata*. Two gland types are present, stalked and sessile. The stalked glands bear a muco-polysaccharide secretion droplet, and are concerned with capture of the prey; the sessile glands are specialised for digestion. In unstimulated glands of both classes, acid phosphatase, esterase and ribonuclease activity is associated with the anticlinal walls of the head cells, which have a characteristic spongy inner surface, comparable with that of transfer cells. Acid phosphatase and esterase activity was also detected in the vacuoles of the head cells of the sessile glands. Substrate film tests showed that amylase is readily released from the stalked glands but not from the sessile ones, while in contrast proteolytic activity is mainly associated with the sessile glands.

On stimulation by suitable nitrogenous materials, the glands begin to secrete fluid onto the leaf surface within 1 hr. During the process the enzymes held in the spongy walls are discharged, and activity is also lost from the intracellular sites in the sessile glands.

Digestion on the leaf surface and resorption of the products has been followed autoradiographically after feeding of ^{14}C-labelled protein. Within 2 hr, digestion products enter the leaf, and move towards the margin in the vascular system. Movement out of the leaf begins within 12 hr. Microautoradiographs showed a concentration of products around the bases of the sessile glands and in the cells of the gland head, showing that these glands are involved in resorption as well as secretion.

A possible mechanism of gland function is discussed.

Introduction

The suggestion that plants of the genus *Pinguicula* were carnivores was made first by Darwin (1875), who confirmed the hypothesis in a series of tests on the excitation and secretion of the leaf. He found that an "acid ferment" was produced freely after stimulation with various organic and inorganic nitrogenous substances, but that no secretion was provoked by water and such inert materials as glass. With his son Francis, Darwin (1878) showed that "feeding" of the leaves of another

insectivorous genus, *Drosera*, with suitable substrate increased the reproductive capacity. These results were confirmed by Oosterhuis (1927), Behre (1929) and Oudman (1936), and more recently they have been extended to *Utricularia* (Pringsheim and Pringsheim, 1967; Harder, 1963, and Sorenson and Jackson, 1968), and to *Pinguicula* itself (Harder and Zemlin, 1967, 1968).

In spite of the evidence of the nutritional value of carnivory, there has been some disagreement about the digestive capacity of leaf-gland secretions (Lloyd, 1942; Schmucker and Linneman, 1959). Morren (1875), Tischutkin (1889) and Olivet and Mirimanoff (1940) considered that the amount of enzyme secreted by *Pinguicula* leaves was so slight that it could hardly account for the digestion of captured insects, and concluded that the so-called "digestion" was really a decomposition by bacteria.

Earlier biochemical work on *Pinguicula* was handicapped by the inavailability of methods of sufficient sensitivity for detecting leaf enzymes, although Dernby (1927) reported evidence of a "tryptase" and possibly also a peptidase in expressed leaf sap and gland secretions collected in glycerine. The evidence for the production of digestive enzymes by other insectivorous genera is much stronger (*Nepenthes* and *Sarracenia*, Lloyd, 1942; *Nepenthes*, Matthews, 1960; *Nepenthes* and *Dionoea*, Lüttge, 1963, 1964, 1965a, 1965b, 1966a, 1966b).

In none of the work on insectivorous species have the enzyme secretions been traced to their intracellular sources, and for this high-resolution cytochemical methods are required. Such methods have recently been applied for the localisation of pollen-borne enzymes (Knox and J. Heslop-Harrison, 1969, 1970a) and in this paper we report the use of similar methods to establish unequivocally which cells of the leaf are concerned in enzyme secretion, and to follow their behaviour on stimulation and during the digestive process.

Materials and Methods

a) Plant Material

Most of the experiments were carried out on *Pinguicula grandiflora* Lamck., originally obtained from wild populations in the counties of Cork and Kerry, Ireland. Clones have been maintained in cultivation for more than 10 years using the methods previously described (Y. Heslop-Harrison, 1962). Plants were grown in a peat-sand mixture, with distilled or de-ionised water supplied only by capillarity from below. The natural pH established in these conditions is 4.7–5.2. Since the hibernaculae of the Irish ecotype require vernalisation at 0.5–1° for at least four weeks, batches could be brought into active growth whenever required. Greenhouse-grown plants were allowed to feed on the available insects. Plants grown in growth chambers during the winter languished unless fed artificially, and for this purpose *Drosophila* were eminently satisfactory. In some experiments, *P. vulgaris* L. and *P. lusitanica* L., of Irish origin, were also used. Tests were also made on a succulent-leaved member of the genus, *P. caudata* Schlecht. (*P. bakeriana* hort., Sander), a native of Mexico.

b) Gland Stimulation

The leaves of plants intended for cytochemical work were guarded from contact with any possible stimulants until needed. Secretion was induced as required by the following stimuli: (1) small, live diptera; (2) small particles of raw meat; (3) 2% monosodium glutamate; (4) 10% commercial soy sauce, and (5) 10% hydroxylamine. In some experiments a mush of macerated cockroach was tested as a stimulant, but this usually caused cell damage.

c) Preparation of Material for Cytochemical Tests

Fresh leaf sections for enzyme localisation were prepared by methods developed by Knox and J. Heslop-Harrison (1969, 1970a) for use with pollen. Plates of a medium containing 15% gelatine, w/v, and 0.8% dimethyl sulphoxide were prepared, and a hole melted to receive fragments of leaf, cut at a width of 4–5 mm with minimal disturbance of surface glands. After setting, a block containing the sample was rapidly frozen on the quick-freeze block of a cryostat. Transverse and paradermal sections were cut at 2–8 μ at −15°, thawed on glass slides and air-dried at room temperature. The sections were stored at 2–5° in a dry atmosphere until required for enzyme tests.

Although observations on sectioned leaves were valuable for verifying certain points concerning enzyme localisation, much more information was obtained using whole mounts of leaf fragments. The usefulness of this method depends upon the fact that the leaves of most of the species of *Pinguicula* used are very thin, with only three or four layers of loosely packed, thin-walled mesophyll cells and the two epidermes. Against this background, the surface glands are quite easily distinguished. Leaf segments were cut directly on to glass slides. For phosphatase, esterase and ribonuclease tests, the fragments were inverted directly into the reaction mixtures in small watch glasses or in cavity slides. After the completion of the reaction, the material was rinsed free of reagents, and fixed in 2.5% glutaraldehyde buffered at pH 7.0 in 0.1 M phosphate buffer at room temperature, usually overnight.

Material fixed this way was viewed directly, or dehydrated for permanent mounting. Because of the delicacy of the gland cytoplasm, it is easily damaged during dehydration; a series of alcohols graded in 10% steps was therefore used, and the process phased over 12–24 h. Clearing was completed in cellosolve before mounting in balsam. For all the enzyme localisation methods described below, the reaction products were found to be stable during this procedure, and no fading has been observed over periods of more than a year.

Handling of the material for substrate film tests for protease and amylase activities is described in the following section.

For cytochemical tests after stimulation, the prey was not removed from the leaf, but the whole section of lamina was cut out, including, for the purpose of comparison, zones of unstimulated glands. The fragments obtained were handled carefully to prevent any accidental stimulation or smearing of the secretions, but no attempt was made to remove the secretion until it was rinsed off in the reaction media. Because the aim in most of the enzyme tests was to follow the behaviour of the glands, enzyme activity in the secretion was not usually studied. The exception was the case of esterase, where the use of the fluorogenic substrate, fluorescein diacetate, made it possible to follow the secretion process with very little disturbance of the glands or materials on the leaf surface.

d) Enzyme Localisation

Acid Phosphatase. Two of methods of Barka and Anderson (1962) which had proved to give excellent localisation in pollen studies (Knox and J. Heslop-Harrison, 1970a) were adopted, using as substrates α-naphthyl acid phosphate and naphthol AS-BI or AS-TR phosphates (Nutritional Biochemicals Corp., Cleveland). The first was used in 0.1 M veronal acetate buffer at pH 6.0 with hexazonium pararosanilin as a coupling agent. The red-brown azo dye produced by the coupling reaction after cleavage of the substrate proved to be very stable in tests made with leaf fragments bearing whole glands, but there was evidence of some diffusion with sectioned material. Naphthol AS-BI and AS-TR phosphates were used as substrates in a similar reaction mixture at pH 5.0, with hexazonium pararosanilin and Fast Garnet GBC as coupling agents. The localisation obtained with these substrates agreed closely with that seen with α-naphthyl acid phosphate.

For both methods the following controls were run: (a) standard reaction mixture, less substrate; (b) leaf fragments pre-incubated for *ca.* 5 min in 0.01 M NaF before incubation in a reaction mixture containing 0.01 M NaF; and (c) leaf fragments supported on glass slides heated over a water bath at 90° for at least 30 min before incubation in the complete reaction mixture.

With all controls some colouration of the leaf gland cells was noted, although the remainder of the leaf remained colourless. However, there was never any indication of the reddish, rose, pink or intense mahogany colour of the reaction products formed in test series. It seems likely that the slight colour in the controls resulted from a reaction between the coupling agents and tannins, which could be detected by the ferric sulphate test in gland cell vacuoles.

Non-specific Esterase. The method of Pearse (1960) was used, with α-naphthyl acetate as substrate in a coupling reaction with fast blue B salt or hexazonium pararosanilin in 0.1 M phosphate buffer, pH 6.0–7.4. Substrate was omitted in the controls. Tests for esterase activity were also made using fluorescein diacetate as a substrate, detecting the cleavage product, fluorescein, by fluorescence microscopy. Although precise localisation cannot be obtained by this method because of the solubility of fluorescein, it is retained in cells with intact membranes—the fluorochromatic reaction (FCR) (Rotman and Papermaster, 1966; J. and Y. Heslop-Harrison, 1970). Because of its very high sensitivity, the method is also useful for detecting esterase activity in secretion droplets. As in earlier work with pollen, fluorescein diacetate was used in saturated solution (*ca.* 5×10^{-6} M) in 0.2–0.4 M sucrose (J. and Y. Heslop-Harrison, 1970; Knox and J. Heslop-Harrison, 1970b). For the study of the glands, leaf surfaces were flooded with the medium, and the development of the FCR followed using a mercury-arc source and Bausch and Lomb 7–37 excitation and T2 barrier filters. To detect esterase activity in secretion droplets, small amounts of the reagent were introduced with a hypodermic needle, and the development of fluorescence followed using a low power objective with the specimen uncovered.

Ribonuclease. Procedures based upon the lead salt method of Enwright *et al.* (1965) were used. Difficulties were encountered in applying the original method to pollen, because results with the concentration of lead ions (0.30%) recommended for erythrocytes were variable and unpredictable (Knox and J. Heslop-Harrison, 1970a). No such difficulties were met with the *Pinguicula* leaf glands, and essentially similar localisation was obtained with 0.30% lead ions and with the medium containing 0.12% lead ions used for the pollen wall enzymes. Suitable media were prepared as follows: 30 mg RNA (from *Torula* yeast) were dissolved in 25 ml 0.2 M acetate buffer, pH 5.0, and 10 mg acid phosphatase (wheat germ) added. Lead, as acetate, was added in solution to give final ionic concentrations of 0.12 or 0.30% in a total volume of 100 ml. The milky suspension was not filtered off. Leaf fragments were incubated in the media for 1–3 h. The lead phosphate reaction product was converted to lead sulphide by transferring the fragments to freshly made H_2S water for 2 min.

Controls were run using media without the substrate, RNA, and by pre-incubating leaf fragments in 0.01 NaF for 5 min before incubation in the complete medium containing 0.01 M NaF. In this case, no reaction was observed in any control.

"Amylase" and "Protease". Substrate film methods were used for the localisation of amylolytic and proteolytic enzymes, which for the purposes of this account will be referred to collectively as amylase and protease, respectively. For amylase localisation, 1% soluble starch films were prepared on microscope slides and air dried. Leaf fragments were then pressed into contact with the film, firmly enough to give adhesion, but not so heavily as to burst the cells of the gland heads. The mucilage droplets of the stalked glands are dispersed by this process, but this does not seem to affect the resolution of the method. The mounts were incubated for 30 min in a moist atmosphere, and the leaf fragments were removed. The films were then laved gently to remove adherent material and digestion products, and the film stained with iodine-potassium iodide. For protease localisation, 1% gelatine films were prepared, and leaf fragments applied as with the amylase method. After laving, the films were stained with 1% nigrosin. Good localisation was also obtained using the processed colour film method of Fratello (1968), and also, as is described below, as an accidental by-product of the micro-autoradiography.

e) Tracer Studies of Protein Digestion

Digestion and the movement of products into the leaf was followed using ^{14}C-labelled protein (*Chlorella* protein-^{14}C(U), denatured; Radiochemical Centre, Amersham). Fresh, unstimulated, fully expanded leaves of matched size on a series of actively growing plants were fed with the labelled protein by placing a single needle-point load to one side of the mid-rib in the upper one third of the lamina. Leaves were then excised at intervals of 2, 4, 8 and 12 h, placed between filter paper sheets and dried with minimal disturbance under pressure on a hot plate at 80°. The *Pinguicula* leaf flattens to paper thinness with this treatment. The drying paper absorbs much of the gland secretion in the immediate neighbourhood of the protein particle, and with it digestion products still on the leaf surface. There is no reason to suppose that there is much displacement of the tracer within the leaf away from the vicinity of the particle, since the drying is very rapid.

Macroautoradiographs were prepared using Kodak X-ray film, with an exposure of 6 days.

The entry points of the digestion products into the leaf were of interest, and in an attempt to detect these, advantage was taken of the thinness and high transparency of the dried lamina to apply a novel method of whole leaf micro-autoradiography. The dried leaves were mounted on gelatine subbed microscope slides without moistening, and then dipped in Ilford L4 Nuclear Emulsion diluted 3 times and liquified at 35°. Surplus emulsion was drained away as rapidly as possible, and the mount dried immediately in a stream of warm air. After 12 days' exposure, the emulsion was processed, and the whole preparation, leaf with applied emulsion, was dehydrated through an alcohol series, cleared in xylene and mounted in balsam.

It was supposed that the drying at 80° would inactivate proteases in the leaf glands, but this was not always so. The unexpected and often dramatic result was that the photographic emulsion was itself sometimes digested over the gland heads in the less stimulated parts of the leaf, providing an unplanned extension of the substrate film method (Fig. 26). This digestion must have occurred during the very brief period between the application and drying of the emulsion, or perhaps more probably during processing.

f) Electron Microscopy

Leaf material for *transmission* electron microscopy was prepared by standard procedures, using as fixatives either 2% $KMnO_4$ at room temperature for 2 h, or 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, at room temperature for 4–8 h followed by OsO_4 at 1% in the same buffer for 3–4 h at 3–5° C.

The surprisingly successful application of *scanning* electron microscopy to fresh leaves of *Pinguicula* has been described elsewhere (Y. Heslop-Harrison, 1970). Taking advantage of the high pumping capacity of the instrument (Cambridge Instruments Stereoscan Mk II a), it was found possible to view freshly cut, uncoated, fragments of the lamina, about 2 by 3 mm, for periods of 4–5 min after the operational vacuum was attained without any noticeable change of surface features. Unstimulated and stimulated leaves were examined in this manner.

Observations

a) Gland Cytology and Fine Structure

The stereoscan micrograph of Fig. 1 provides a general view of the leaf surface topography of *P. grandiflora*. Two classes of glands are present, stalked and sessile; according to Goebel (1891) they are homologous with trichome hydathodes, having the same general structure. Towards the leaf margins and at the leaf base the structure is modified, and types transitional to more normal trichomes appear. The cellular organisation is shown in Figs. 2 and 3, where the cells are named according to the terminology of Klein (1883).

A typical *stalked gland* consists of a large basal cell, replacing an epidermal cell, a stalk cell, and a columellar cell supporting the glandular head. The basal cell is thicker walled than adjacent epidermal cells, and this gives the mechanical strength required to support the secretion droplet carried on the glandular head (Fig. 1). The droplets are easily visible to the naked eye, giving the characteristic glistening or greasy

Fig. 1. Scanning electron micrograph of the fresh, uncoated leaf surface of *P. grandiflora*, taken within 5–8 min after introduction into the vacuum of the microscope. The two upper stalked glands still carry undried mucilage globules, but the mucilage is drying from the gland in the foreground, and the gland head is becoming visible. The large basal cells and the entasis of the stalk may be seen in the stalked glands. The more numerous sessile glands are sunken in slight depressions of the epidermis, and small secretion droplets may be seen on some. × *ca.* 350

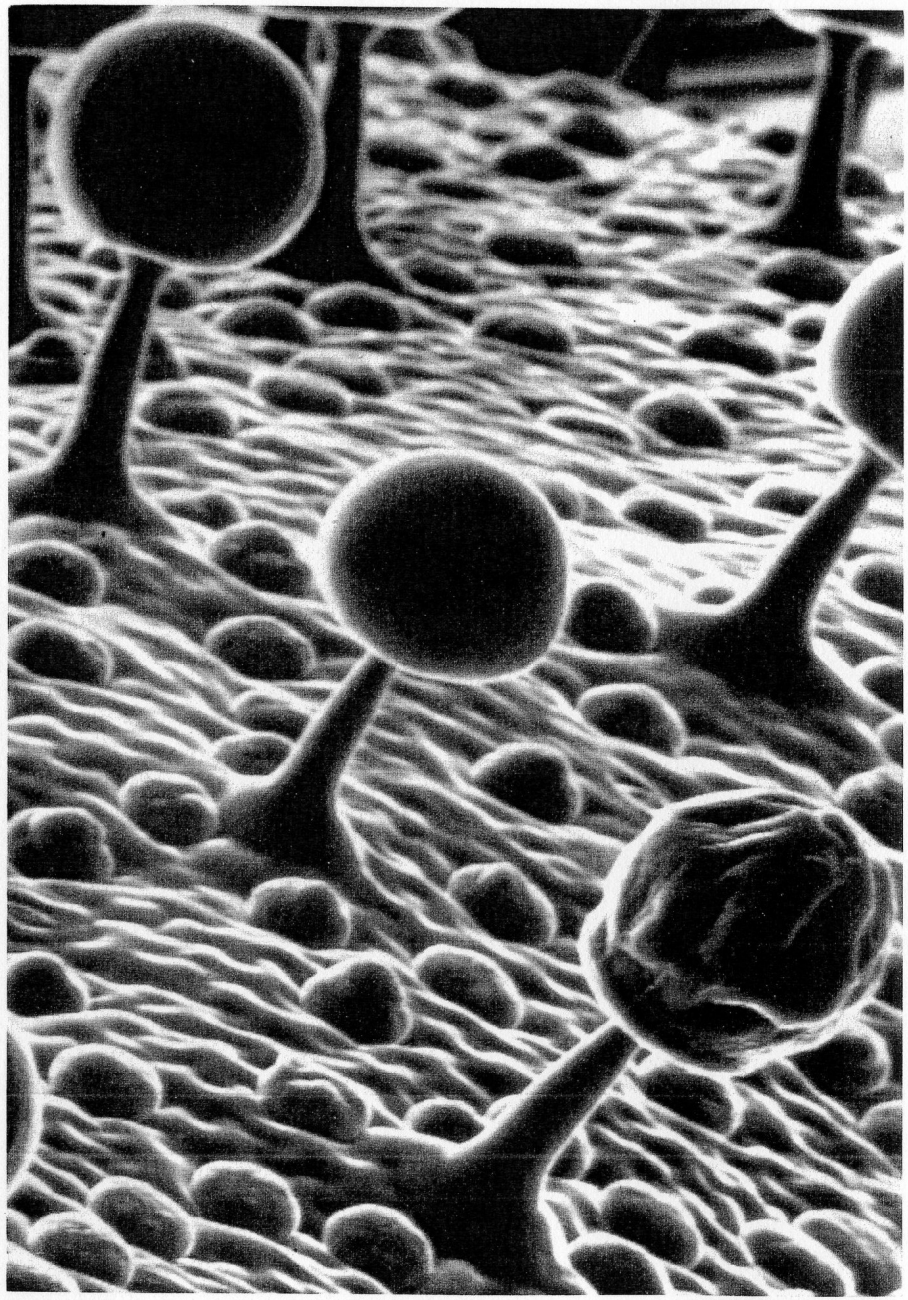


Fig. 1

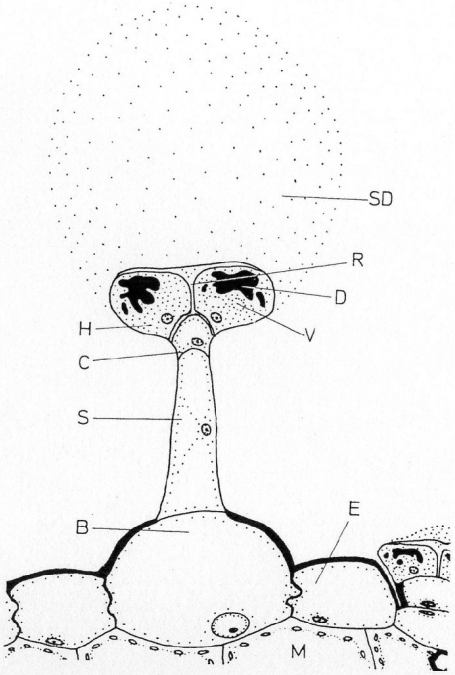


Fig. 2

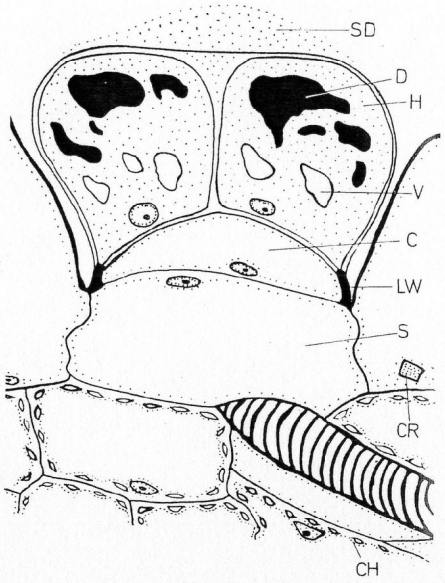


Fig. 3

Fig. 2. Diagram of a section of the upper surface of the leaf of *P. grandiflora* showing the structure of a typical stalked gland, with a portion of a sessile gland to the right. *SD* secretion droplet; *H* head cell; *C* columellar cell; *S* stalk cell; *B* basal cell; *E* epidermal cell; *M* mesophyll; *R* radial wall of head cell; *D* densely staining vacuole-like inclusions; *V* vacuoles. × *ca.* 250

Fig. 3. Diagram of a typical sessile gland of *P. grandiflora*. *CH* chloroplasts; *CR* crystalline inclusion; *LW* thickened lateral wall of the columellar cell; other lettering as in Fig. 2. × *ca.* 1000

appearance to the leaf. The heads of glands in the centre of the lamina consist of 16 (occasionally 32) cells, radiating symmetrically from the columella; towards the leaf margin the number of cells in the head is lower, 8 or 16.

In the unstimulated gland, the nuclei of the head cells lie appressed to the wall of the columellar cell. One or two large vacuoles occupy the upper part of the cells, so that the bulk of the cytoplasm also lies adjacent to the columellar cell. The mitochondria of the mature gland are large, with well developed cristae. The plastids are unexceptional in size and development, and ribosomal endoplasmic reticulum (ER),

while present, is not so prominent a feature of the stalked gland cells as it is in the sessile glands. On the other hand, dictyosomes are conspicuous, with numerous associated vesicles suggestive of intense activity.

Schnepf (1961, 1963) has interpreted this as indicating that the Golgi apparatus is responsible for the secretion of the large mucilage droplet borne by the gland head. Analyses of the contents of the secretion are not available for *Pinguicula*; however, Schnepf (1963) has reported on the *Fangschleim* of another insectivorous genus, *Drosophyllum*, which is evidently quite similar to that of *Pinguicula*. The gummy material was found to be a polysaccharide which yielded galactose, arabinose, xylose and rhamnose on hydrolysis; ascorbic acid and—rather unusually—gluconic acid were also found to be present. No ninhydrin-positive substances were detected.

The secretion process involves the passage of materials outwards through the walls of the gland head cells, and the characteristic wall adaptation described by Gunning *et al.* (1968) for "transfer cells" is conspicuously present. The radial walls of the head cells possess a thick, spongy surface, composed of fingers, ribbons or plates of a material giving a positive reaction with the periodic acid-Schiff's reagent extending inward from the primary wall, and interdigitating with the cortical cytoplasm (Fig. 4). The spongy wall is thickest in the central region, tapering off outwards toward the parietal walls and inwards towards the columella cell.

The *sessile glands*, seen in surface view in Fig. 1 and in diagrammatic representation in Fig. 3, consist of a basal cell, sunken slightly below the level of the epidermis, and a columellar cell supporting the capital of 2–8 cells. As Haberlandt (1884) first noted, the columellar cell of the sessile gland has a rather strongly cutinised lateral wall, the thickening being somewhat reminiscent of the Casparian strip of an endodermal cell. As mentioned in a later paragraph, this thickening may have some significance in connection with secretion and resorption.

The epidermis is slightly depressed near each sessile gland, which means that the head cells will be immersed very readily in fluid droplets formed on the leaf surface. In the unstimulated leaf, the sessile glands do not secrete the quantity of fluid found on the stalked glands. However, stereoscan micrographs of fresh leaves show that they do retain a small droplet in a central position (Fig. 1).

In the unstimulated sessile glands, the nuclei occupy the positions seen in Fig. 3. The cytoplasm of each head cell is concentrated mostly near the columellar cell, and the upper volume is occupied by large vacuolar inclusions, the contents of which blacken with $KMnO_4$ and OsO_4 and are responsible for the tannin reaction mentioned above. The mitochondria of the head cells are large and often pleiomorphic or even moniliform, and the same kind of ramification is seen in the plastids, which are often very large, up to 4 μ across, including extended processes.

Rough-surfaced endoplasmic reticulum is very well developed and, as first shown by Vogel (1960), sheaths of ER enclose each plastid, following precisely every ramification of the plastid envelope. Where the ER invests a plastid, ribosomes are borne only on the outer surface of the outer membrane, and not between the inner membrane and the outer membrane of the plastid envelope.

The radial walls of the head cells of the sessile glands are spongy, as in the head cells of the stalked glands, having the characteristics of those of transfer cells (Fig. 4).

The dimorphism of the leaf glands of *Pinguicula* was described by Darwin (1875), who reported that both classes were secretory. Haberlandt (1884) recognised a functional distinction, suggesting that the stalked glands are primarily responsible for insect capture, while the sessile glands are concerned with digestion of the prey and absorption of the products. Most subsequent authors have accepted this interpretation (Lloyd, 1942).

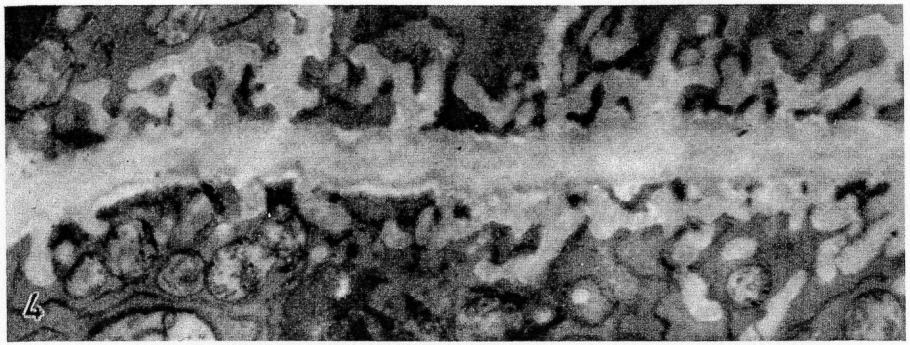
b) Enzyme Localisation in Unstimulated Leaves

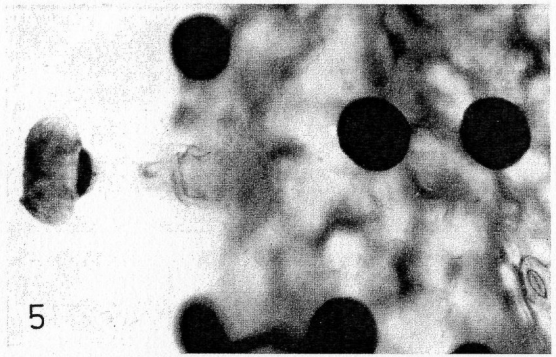
Darwin (1875) showed that secretion began in as short a period as 40 min after the application of meat to the leaf surface, and his experiments also suggested that digestion must begin almost as soon as the secretion comes in contact with suitable substrate. The implication is that the digestive enzymes are stored in the gland cells, and pass out with the secretion on stimulation. The cytochemical methods for acid phosphatase, esterase and ribonuclease are capable of giving good intracellular localisation, and we have used them to identify the main sites of enzyme storage in unstimulated leaves.

The only cells of the leaf in which substantial enzyme activity was detected were those of the glands, and all three enzymes were present in both sessile and stalked glands. However, so far as the density of the reaction product can be taken as a guide, it is clear that much the greatest concentration is present in the sessile glands (Figs. 5 and 7).

Acid Phosphatase. Activity is present throughout the head cells of the sessile glands (Fig. 5), but the heaviest deposition of the reaction product was always associated with the spongy radial walls of the head cells. There was no evidence of activity being associated with particulate structures in intact cells, and the uniform distribution of the reaction product in the upper part of each head cell suggested that the enzyme was present in the vacuole-like inclusions. Observations on freeze-sectioned glands supported this interpretation. The residual activity in sections was invariably found in the spongy lateral walls of the head cells, with little or none in the cytoplasm. The implication is that the enzyme is lost from the cut cells.

The acid-phosphatase activity of the stalked cells was found to be strikingly concentrated in the radial walls of the head cells (Fig. 6), and there was no indication of storage within the cell comparable with that seen in the sessile glands. In a few cases, reaction product was visible in the lower wall of the columellar cell (Fig. 5).





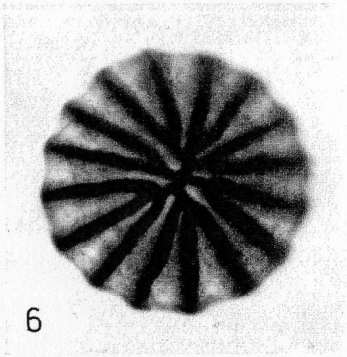
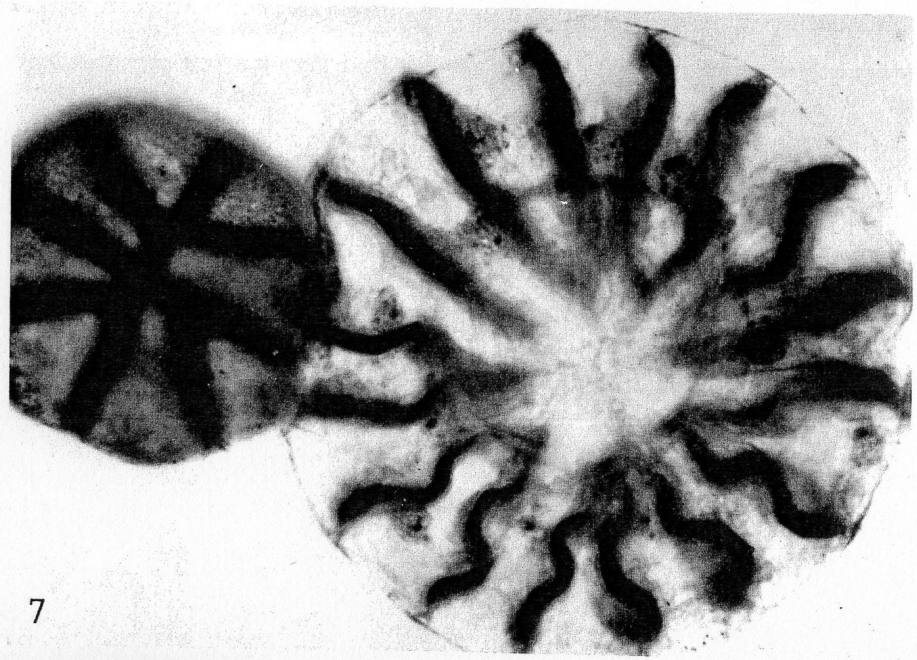
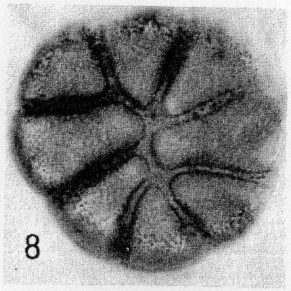


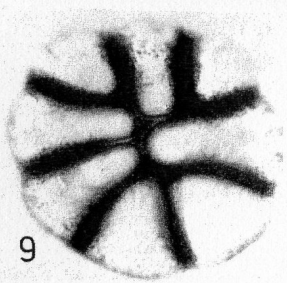
Fig. 4. Electron micrograph of the central area of the anticlinal wall separating two head cells of a sessile gland of *P. grandiflora* showing the interdigitating cytoplasmic embayments and wall processes. Fixation: 2% $KMnO_4$. × *ca.* 28000

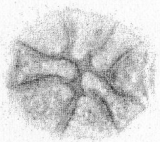
Fig. 5. Portion of an unstimulated leaf of *P. grandiflora*; α-naphthyl acid phosphate—pararosanalin reaction for acid phosphatase. The upper part of the leaf is bent over, and a stalked gland is seen in side view; in this gland there is an unusual accumulation of reaction product in the wall between the columellar cell and the stalk cell. There is a heavy accumulation of reaction product throughout the head cells of the sessile glands, although the concentration in the anticlinal walls can still be discerned. Each sessile gland is surrounded by 5–7 radially arranged epidermal cells, which probably provide one of the sources of fluid during secretion. × *ca.* 200

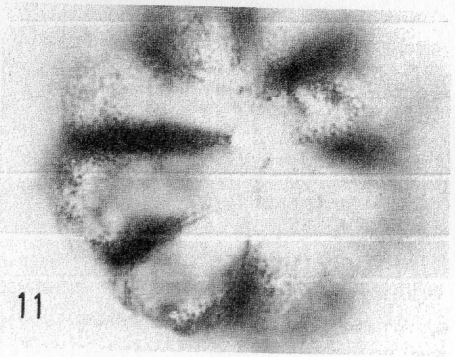
Fig. 6. Surface view of the head of an unstimulated stalked gland of *P. grandiflora*, showing the heavy concentration of acid phosphatase activity associated with the 16 anticlinal walls; α-naphthyl acid phosphate—pararosanalin reaction for acid phosphatase. × *ca.* 400

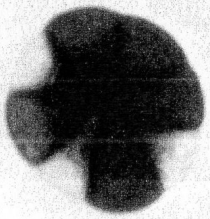












Figs. 7–12

Esterase. The distribution of esterase activity matched that of phosphatase quite closely, and again there was substantial activity within the protoplasts of the head cells of the sessile glands, as well as in the spongy walls (Figs. 17 and 18). Activity in the stalked glands was restricted to the radial walls of the head cells.

The sessile glands showed a rather unusual fluorochromatic reaction when supplied with the esterase substrate, fluorescein diacetate. Fluorescence quickly appeared in the radial walls, and there was an initial build up in the adjacent cytoplasm. Thereafter fluorescein was rapidly lost from the gland, with an accompanying increase in the fluorescence of the medium. The early cleavage of the substrate in the walls is to be expected from the concentration of enzyme seen there with the α-naphthyl acetate reaction. The absence of a true fluorochromatic reaction in the cytoplasm indicates that the plasmalemma is initially—or quickly becomes—rather freely permeable to fluorescein, which is accordingly not accumulated intracellularly.

Ribonuclease. The method adopted for this enzyme gave precise and entirely reproducible localisation. In all cases, activity was principally in the spongy anticlinal walls of the head cells in both sessile and stalked glands. As Fig. 7 shows, the density of reaction product is greater in the sessile glands, where some activity is also associated

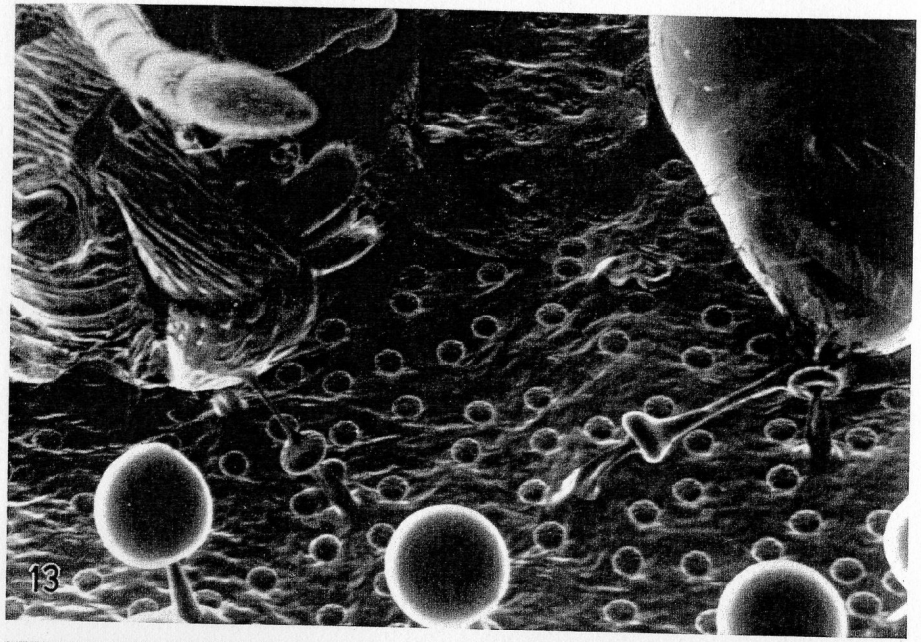
Fig. 7. Neighbouring sessile and stalked glands from an unstimulated leaf of *P. grandiflora*, RNase reaction. Strong ribonuclease activity is associated with the spongy anticlinal walls, but that in the sessile glands is even greater. Reaction product is also present over the inner surface of the periclinal walls of the head cells of the sessile gland. Neither gland shows cytoplasmic activity, but that associated with the nuclei may be seen in the head cells of the stalked gland. × *ca.* 1000

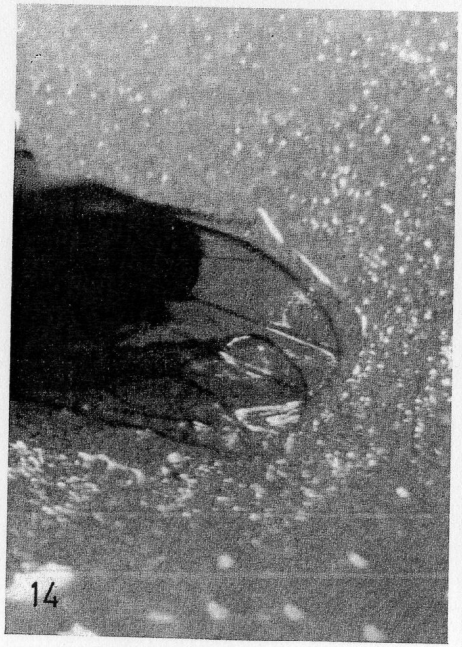
Figs. 8 and 9. A slightly stimulated sessile gland of *P. grandiflora*, two focal planes; RNase reaction. Fig. 8, focussed at the level of the outer walls of the head cells, shows the granular deposition of the reaction product on the inner surface. In Fig. 9, focussed in the centre of the head cells, the strong concentration of reaction product in the spongy parts of the anticlinal walls may be seen. × *ca.* 700

Fig. 10. Unstimulated sessile gland of *P. grandiflora*, RNase control + 0.01 M NaF as inhibitor. The radial walls are visible, but there is no reaction product. Controls without substrate were similarly negative. × *ca.* 400

Fig. 11. Partially stimulated sessile gland of *P. grandiflora*, RNase reaction. The enzyme has evidently drained entirely from the central part of the outer walls, and has also left the less spongy regions of the anticlinal walls. × *ca.* 1000

Fig. 12. Partly stimulated sessile gland of *P. grandiflora*, α-naphthyl acid phosphate—pararosanalin reaction for acid phosphatase. Five head cells show activity, whilst three have evidently discharged the vacuole-held enzyme. The circular outline of the columellar cell, containing small amounts of enzyme, is discernible below the gland head. × *ca.* 550





Figs. 13–15

with the outer walls of the head cells. This may be seen in Figs. 8 and 9 which show reaction product at two focal planes, one on the gland surface and the other at the level of the centres of the cells. The focal plane in the control seen in Fig. 10 is at a level corresponding to that of Fig. 9.

The only intracellular site of appreciable ribonuclease activity in mature glands was in the nuclei, and particularly in the nucleoli, as may be seen in the stalked gland head in Fig. 7.

c) Enzyme Activity in Developing Glands and Hydathodes

A study of the development of enzyme activity in the leaf glands is in progress, but some of the salient facts require mention here. There is no wall-associated phosphatase, esterase or ribonuclease activity in differentiating glands in which cell division is still in progress. The formation of the spongy radial walls does not begin until the completion of divisions in the head, and it is not until wall thickening is essentially well advanced that associated enzyme activity becomes detectable. Leaf glands near the leaf margin show imperfect development of the head, and the cells evidently do not have the full enzyme complement; of the three tested, acid phosphatase and esterase activity were detected, but not ribonuclease. Similarly, the hydathodes carry acid phosphatase and esterase, but lack ribonuclease. Enzyme activity is very slight in the hairs of the basal part of the midrib, although these are undoubtedly homologous with the glands.

d) Effects of Stimulation

The capture of an insect is accomplished by the mucilage held by the stalked glands. This adheres immediately on contact with any part of the prey, and when drawn out, it sets into strong cables, as seen in the stereoscan micrograph of Fig. 13. In this early stage there is no secretion by the sessile glands.

Fig. 13. Scanning electron micrograph of a portion of the leaf of *P. grandiflora* bearing a captured ant. At the lower margin, three stalked glands remain undischarged. Where the insect has come in contact with stalked glands, the secretion has been drawn out into cables. × *ca.* 100

Fig. 14. *Drosophila* on the leaf of *P. grandiflora* after 6–7 hr. The wings and body are partly submerged in the secreted fluid, which has marked detergent properties. × *ca.* 40

Fig. 15. Leaf margin of *P. grandiflora* unrolling after the digestion of a fly. The gland secretion has been completely resorbed, and the fly is desiccated. The secretion shows no chitinase activity, and the exoskeleton is intact. × *ca.* 25

The induction of secretion was meticulously investigated by Darwin, and with regard to the general phenomenology little has been added to his observations in the period since the publication of *Insectivorous Plants* in 1875. While mere mechanical stimulation provokes no additional secretion, Darwin found that a copious outflow could be induced on excitation with insects, meat, albumen, gelatine, casein, milk, various plant proteins and ammonium carbonate. Whereas the secretion of the unstimulated leaf was not very acid, Darwin noted that that induced by contact with nitrogenous materials was markedly acid. We have found the surface fluid of unstimulated leaves, which includes the mucilage from the stalked glands, to have a pH of 5.0–5.4, and the secretion induced by meat to lie between pH 3.1 and 3.4.

Timing of Gland Discharge. Darwin reported that the shortest period for the appearance of the acid secretion after stimulation of a fresh leaf with meat was 40 min. With natural prey such as small diptera secretion does not begin so rapidly, but if contact with the leaf surface has been good, accumulation of fluid may be seen in 2–3 h. Thereafter a pool is formed, often deep enough practically to immerse the prey. The secretion has marked detergent properties, and quickly wets normally water-resistant surfaces of the exoskeleton (Fig. 14). Resorption begins after a few more hours, and the dry undigested remnants of the prey are left on the leaf surface (Fig. 15).

The appearance of esterase activity in the leaf-surface fluid was followed using fluorescein diacetate as a substrate after placing meat particles on fresh, unstimulated parts of the lamina. The first evidence of activity was noted after one hour, when the particle was moist but not immersed. After 2 h, when the particle lay in a pool of secretion, intense activity could be detected.

Sources of the Secreted Enzymes. The effects of stimulation on the enzymes held in the head cells of stalked and sessile glands was followed using the various fluid and solid stimulants. All agents brought about a partial or complete discharge from both stalked and sessile glands.

The response to natural solid prey, exemplified in the experiments with *Drosophila*, may be interpreted as follows. Secretion begins from those stalked and sessile glands first making contact with the body of the fly. As this secretion accumulates to form a pool, it immerses more and more glands, which in turn are provoked to secrete. The limits of the pool are determined by the size of the prey and the local topography of the leaf. With a small object, resorption may begin quite rapidly, and the pool then ceases to extend. With larger objects, the secretion is probably limited by the capacity of the leaf to provide water, although as noted by Darwin the secretion may be so copious as to run to the midrib and then even off the leaf.

The field in Fig. 16 covers the whole width of the secretion pool around a single *Drosophila* after the reaction for acid phosphatase with α-naphthyl acid phosphate as substrate. The sessile glands in the area

of the pool are discharged, although many show a residue of activity in the spongy radial walls of the head. The stalked glands around the insect are also discharged, but towards the outer limit of the pool many are undischarged, although surrounded by discharged sessile glands. These have escaped from stimulation because their heads have been held above the level of the secretion pool.

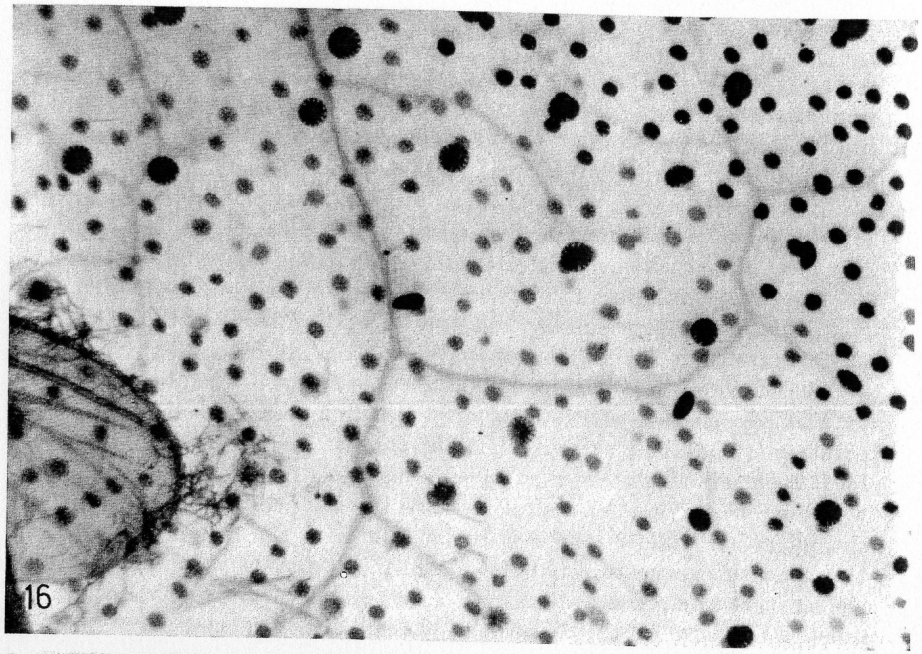
In stimulated areas of the lamina tested for acid phosphatase and esterase, sessile glands may often be seen in the vicinity of the prey in a partially discharged state, but stalked glands are rarely seen in intermediate conditions. It may be inferred from this that the stalked glands discharge very rapidly and completely, probably because they do not store these enzymes internally.

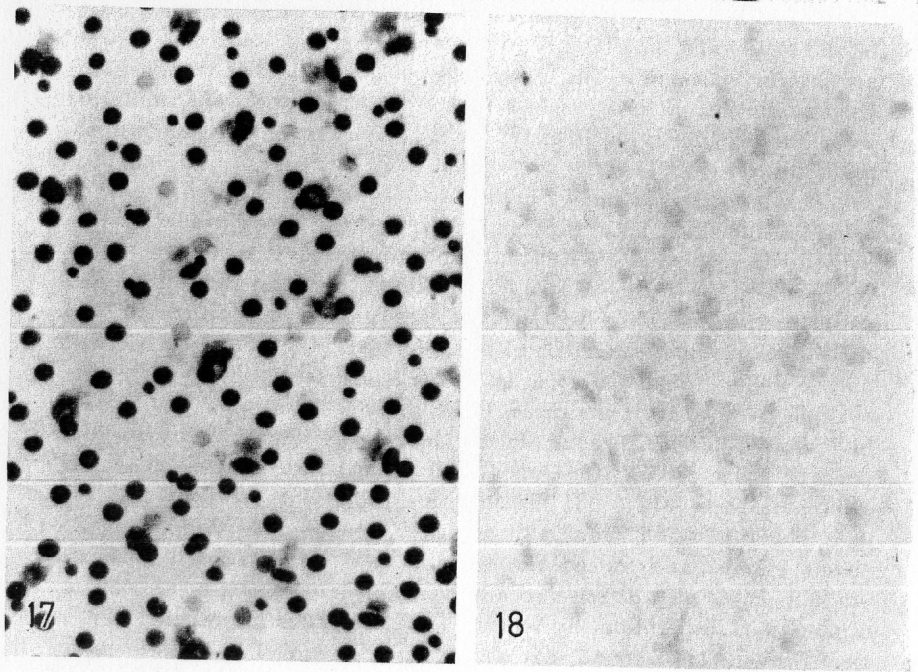
The enzymes held within the head cells are typified in this study by acid phosphatase and esterase, and it is not possible to specify the pathways they take on passage out of the cells. Aspects such as that of Fig. 12 suggest that there may be a direct discharge through the outer wall, and this is perhaps supported by the fact that activity in the spongy radial walls is the last to be lost from the glands (Fig. 16). However, this sequence could also mean that enzymes of the head cell vacuoles are pumped into the radial walls, which remain charged to the last because they are the channels of passage out of the gland.

As already mentioned, ribonuclease is not held within the protoplasts of the head cells, but mainly in the spongy radial walls and in what are possibly pockets in the outer wall of the sessile glands (Figs. 7–9). On stimulation, activity is lost first from the outer walls, and then from the less spongy parts of the radial walls, remaining longest in the thickest zone (Fig. 11).

Location of Amylase and Protease Activity. Because the tests for these enzymes depended upon the use of extracellular substrates, they were carried out with what were, strictly speaking, stimulated leaves.

Surprisingly, starch substrate films showed that the greatest amylase activity was associated with the stalked glands (Fig. 23), with very little digestion over the sessile glands. Moreover, the resolution obtained with the best films was such as to suggest that activity was present over all parts of the gland-head cells, stalk cell and basal cell. Taken at its face value, this might suggest that the secretion of amylases and perhaps other carbohydrases is the prerogative of the stalked glands. However, there are other possible interpretations of the result; for example, the starch films may not excite enzyme secretion from the sessile glands, and indeed the amylase activity associated with the stalked glands may be released into the films by pressure at the time of application, rather than by natural secretion. It is interesting to note that Darwin (1875) found that starch particles provoked secretion from





Figs. 16–18

the leaf of *Pinguicula*, but that the secretion was not acid and was incapable of digesting the added starch.

Protein-containing substrate films consistently showed that the greatest proteolytic activity was associated with the head cells of the sessile glands, although digestion was often seen over the heads of stalked glands also. As already mentioned, some of the best demonstrations of the localisation of secreted proteases were obtained with photographic emulsions, and an example is illustrated in Fig. 24.

e) Digestion of Labelled Protein

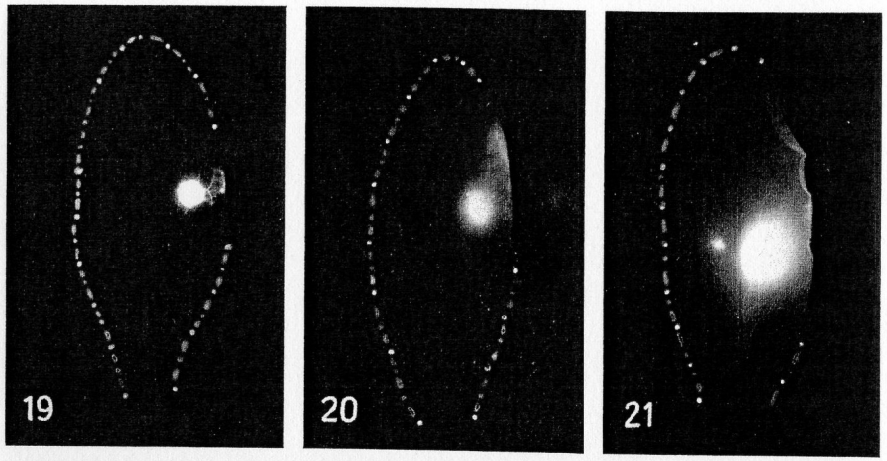
Time Course. The sequence of autoradiographs in Figs. 19–22 shows the localisation of products after 2, 4, 8 and 12 h. It is evident from Fig. 19 that digestion has already begun within 2 h, and that the products have penetrated the leaf, concentrating in the vascular bundles near the site of application of the labelled protein. The first direction of movement within the leaf is always towards the adjacent margin (Fig. 20); thereafter the products move into the midrib (Fig. 21). As Fig. 22 shows, transport in the vascular system is both basipetal and acropetal. In this experiment, digestion products had reached the leaf base and were passing into the axis by 12 h.

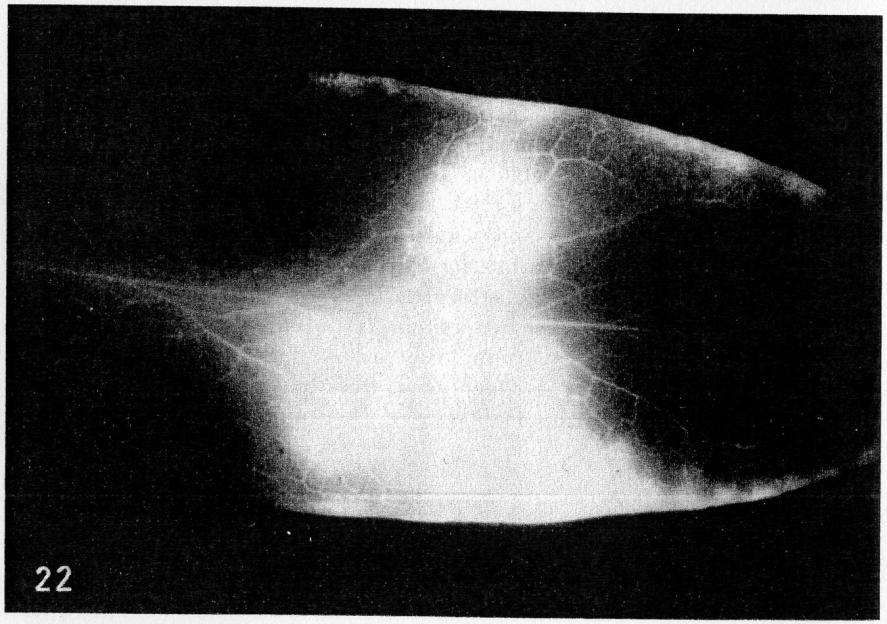
Sites of Uptake. The pools of secretion on the leaf surface were spread somewhat during drying for autoradiography, and the zones of intense radioactivity in the macroautoradiographs of Figs. 19–22 mark these regions and also a surrounding halo where activity present within the leaf cells was sufficient to saturate the emulsion with the exposure given. In the microautoradiographs similarly the emulsion was saturated over much of the surface around the protein particles. However, in areas of lesser activity the silver grain distribution could readily be related to features of the leaf surface. A characteristic disposition

Fig. 16. Whole mount of a portion of a leaf of *P. grandiflora* bearing a captured fly, α-naphthyl acid phosphate—pararosanalin reaction for acid phosphatase. The zone of stimulated glands extends around the fly, the wing of which is seen to the lower left, corresponding in area to the extent of the secretion pool. The glands toward the top right are unstimulated, and show intense activity. While the stalked glands whose heads have been immersed in the secretion pool have also discharged, those towards the margin of the pool which have not been completely immersed have retained enzyme activity. × *ca.* 50

Fig. 17. Portion of an unstimulated leaf of *P. grandiflora*, α-naphthyl acetate—pararosanalin reaction for esterase. Some of the stalked glands have been bent during processing, and the smeared mucilage has stained slightly. Otherwise the enzyme distribution follows that of acid phosphatase. × *ca.* 50

Fig. 18. Control for Fig. 17, complete reaction mixture less substrate. × *ca.* 50





Figs. 19–21. Macroautoradiographs of leaves of *P. grandiflora* excised 2, 4 and 8 hr after feeding with ^{14}C-labelled protein. A needle-point load of protein was placed to the right of the midrib in each case, except that in the leaf of Fig. 21 another small particle has contaminated the lamina on the left. The blade is outlined by the white chain-dot line. The emulsion is saturated over the central area of intense radioactivity in each case. The radial spread of this area is due both to increase in the size of the secretion pool, which is partly drained during drying for autoradiography, and by movement of the digest within the leaf. The early transport to the margin may be seen in Figs. 19 and 20; after 8 hr, activity is moving freely in the vascular system towards both proximal and distal ends of the leaf. × *ca.* 1

Fig. 22. As Figs. 19–21, but leaf excised after 12 hr. Products of digestion have now entered the midrib, and at this time radioactivity can be traced right to the leaf base and out of the leaf. × *ca.* 3

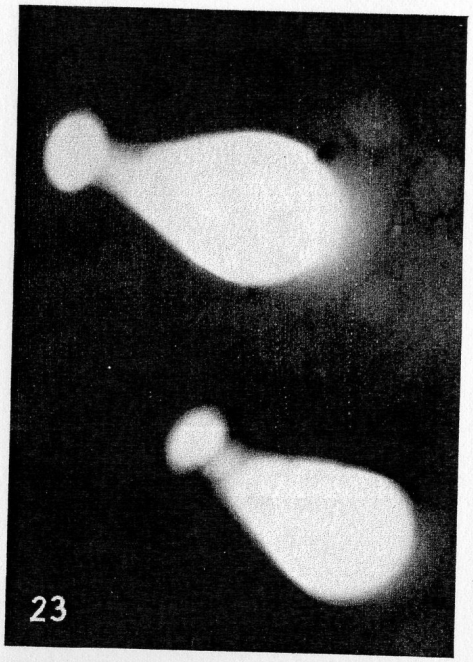
in the neighbourhood of the sessile glands is seen in Fig. 26; here there is a concentration of grains in a ring over the annular pit surrounding the sunken basal cell (compare Fig. 2). This concentration could be an artefact resulting from a greater thickness of emulsion around the periphery of the gland head, but the consistency of the appearance may also be taken to indicate that the pit at the base of the gland forms a collecting vessel for the digestion products.

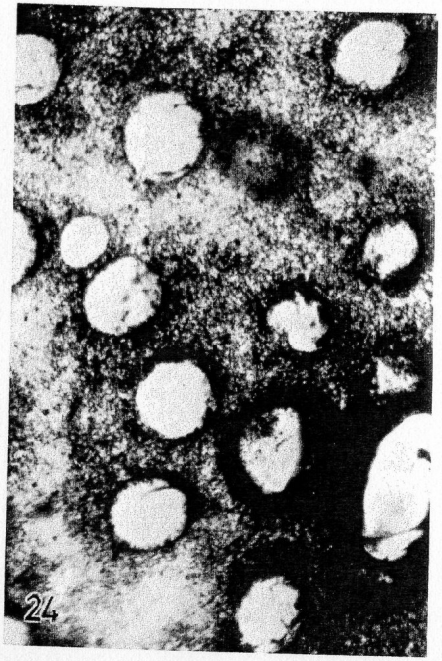
While the emulsion was frequently digested over the heads of the sessile glands (Fig. 24), many were successfully coated. In these cases, the distribution of silver grains often defined the outlines of the head cells, showing some concentration over the spongy radial walls (Fig. 25). It is not easy to envisage a form of artefact which would produce this effect, so it is reasonable to conclude that digestion products do move into the head cells and are retained, for a period at least, in the spongy walls.

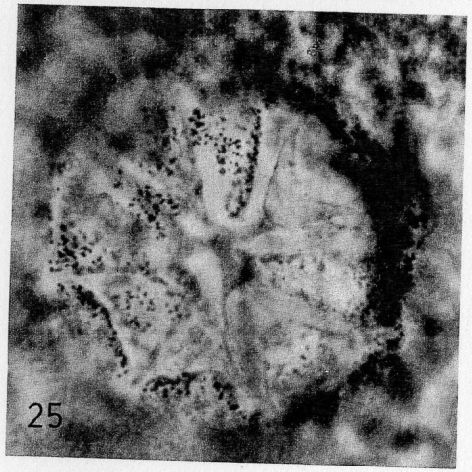
The pathways of movement of the tracer in the leaf were not noticeably better defined in the microautoradiographs than in macroautoradiographs like that of Fig. 22. In both, silver grains followed the course of the vascular bundles, even to the terminations in mesophyll islets. These terminations underlie the sessile glands, and presumably the nett movement is away from the end of the bundle in parts of the lamina flooded with the digest. However, there is no doubt that digestion products are also quickly distributed through the vascular system *into* parts of the mesophyll not directly underlying the secretion pool. It seems likely that the movement seen in the sequence of Figs. 19–21 is related to the pathway of water. This might be taken to suggest that some movement is in the xylem, but the resolution obtained in the autoradiographs was not good enough to establish this point.

Discussion

a) Enzyme Storage Sites. These results establish beyond doubt that the leaf of *Pinguicula* is an effective digesting organ, and that the enzymes concerned in the digestive processes are held in the glands of the leaf surface, from which they are released on stimulation. This interpretation is in essence that of Darwin (1875); the alternative possibility







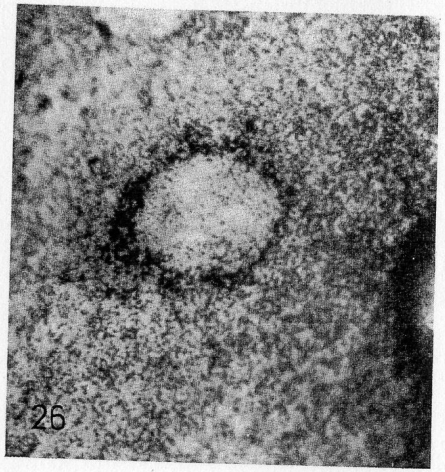


Fig. 23. Amylase activity on the upper surface of a leaf of *P. grandiflora* shown by starch film digestion. The outlines of the appressed and enlarged stalked glands may be seen; the paler grey circles show the location of the sessile glands, over which there has been little digestion. The implications of this result are discussed in the text. One per cent. starch film, post-stained in iodine. × *ca.* 150

Fig. 24. Photographic emulsion digested over the heads of sessile glands of the upper leaf surface of *P. grandiflora*. × *ca.* 200

that digestion on the leaf surface is principally due to bacteria—maintained by various authors up to comparatively recent times—may now be rejected.

It is noteworthy that enzyme activity can be demonstrated in both classes of gland in the unstimulated leaf, although for phosphatase, esterase and ribonuclease the cytochemical results do suggest that activity is higher in the sessile glands than in the stalked. The tests with protein films suggested that proteolytic enzymes are also held in both types of gland. Amylase activity is perhaps primarily associated with the stalked glands, but as mentioned above this cannot yet be asserted positively.

The sites of enzyme storage within the glands have been clearly enough defined by the cytochemical reactions for esterase, phosphatase and ribonuclease. The significance of the spongy anticlinal walls of the head as a repository is not in doubt. Evidently these walls are charged during the later period of differentiation of the head cells, and retain the enzyme content during the life of the gland unless stimuli provoking secretion are received. The enzymes are presumably held in the embayments in the polysaccharide matrix, and if these are all in interconnection—representing ramifications of the cortical cytoplasm of the head cells—the storage sites are not truly extracellular. The situation in not, then, exactly comparable with that seen in the pollen grain, where enzymic proteins are held in the cellulosic intine outside of the plasmalemma (Knox and J. Heslop-Harrison, 1969, 1970). Apart from the storage sites in the spongy wall, phosphatase and esterase activity is also present in the vacuole-like inclusions of the upper parts of the head cells of the sessile glands. The cytochemical technique did not expose ribonuclease activity in these sites, but it is not of course excluded that these inclusions within the protoplast are repositories for other enzymes.

b) Mucilage Secretion and Enzyme Discharge. There is no indication that the secretion droplet carried by the stalked glands of the unstimulated leaf contains enzymes; its role, as postulated long ago by Haberlandt (1884) and others, seems entirely connected with the capture

Fig. 25. Microautoradiograph of the head cells of a sessile gland of *P. grandiflora* after resorption of digest of ^{14}C-labelled protein from the leaf surface. The distribution of silver grains suggests that digestion products have been taken into the head cells. × *ca.* 1 000

Fig. 26. As Fig. 25, but showing concentration of radioactivity in a ring defining the annular pit in which the sessile gland is sunken. This concentration may also be seen in Fig. 25, although the focal plane is somewhat too high to define it perfectly. × *ca.* 400

[illegible] of the head cells gives every support for Schnepf's postulation (1961, 1963) that the mucilage secretion results from dictyosome activity. The implication is that the mucilage is released from dictyosome vesicles into the walls of the head cells and passed through into the droplet as a normal secretory process in the unstimulated gland, while the enzymes are retained within the cell, to be secreted only on stimulation. This duality of function could be accounted for were the pathways different. Phosphatase, esterase and ribonuclease seem to be exclusively associated with the spongy anticlinal walls in the head cells, with no storage in the protoplast (Figs. 6 and 7). Possibly the dictyosome discharge is through the lightly cuticularised parietal walls, a point that could no doubt be determined electron microscopically.

In any event, the mechanism of emission of the enzymes is likely to be quite different from that involved in the mucilage secretion. As Darwin (1875) showed, soluble nitrogenous compounds provoke the release of an acid secretion on to the leaf surface, and this he correctly assumed to be responsible for digestion. While many aspects of the secretion process remain to be explained, the present study has clarified some features. In both stalked and sessile glands, the released enzymes are derived largely from the wall sites: perhaps entirely so in the case of ribonuclease (Fig. 11). There is probably also some release of activity through the parietal walls of the sessile glands for the enzymes with storage sites within the protoplast (Fig. 12).

c) Mechanism of Discharge. The discharge is associated with a copious release of fluid ultimately amounting to many times the volume of the glands (Darwin, 1875). There is good reason to suppose that the greater part of this is secreted from the sessile glands, which are more numerous and which show the more conspicuous structural changes during secretion (Mirimanoff, 1938). Undoubtedly much of the secreted water is derived from neighbouring cells of the epidermis (Fig. 5), and its abstraction may be responsible in part for the incurving of leaf margins near stimulated areas noted by Darwin (1875). However, to account for the volume of flow in a strongly stimulated leaf, it must be supposed that water moves in from elsewhere in the plant. Various anatomical features point to a similarity between the glands with their adjacent tissues and active hydathodes (Stocking, 1956). The stalk cells of the sessile glands are associated with terminal xylem elements (Fig. 3; Fenner, 1904), and water lost during secretion no doubt passes in through this route. As we have seen, the columellar cells above the stalk cells have strongly cutinised periclinal walls, and this must ensure that water entering the head moves entirely through the cytoplasm of this cell [compare, for example, Arisz's (1956) views of water movement in the root]. It cannot be without significance that the same columellar cell is richly populated with large mitochondria possessing well developed cristae. Since presumably the secretory process is energy requiring, these are in a strategic position for providing the ATP required.

If it be accepted that water itself is never actively moved in the plant, then it must be supposed that secretion from the sessile glands of *Pinguicula* depends upon the development of osmotic pressure in the cells of the gland head. A possible interpretation is that stimulation brings about a rapid increase in the concentration of osmoticum in the head cells, causing at first an endosmosis of water drawn from the columellar cell. With increasing turgor in the head cells, leakage to the outside begins through the spongy walls, and the enzymes held there are leached out. Possible immediate return pathways for the fluid released back into the leaf are blocked by the cutinisation of the epidermal cells and of the periclinal wall of the columellar cell itself. As long as the plasma of the columellar cell continues to act as a semi-permeable membrane between the head cells and the stalk cell, water will be drawn from the stalk cell, which in turn will abstract water from the xylem element terminating at its surface. The water supply from the root will thus be directly tapped. This interpretation is in accord with the anatomy; the steps that remain to be verified and elucidated are of course those postulated as occurring in the gland head. Some light may be cast on these by fine-structural studies of the changes in the head cells which immediately follow upon stimulation (Mirimanoff, 1938). Beyond this, information is needed on the receptor sites for the stimulus, and on the nature of the metabolic processes triggered in the head cells which increase the concentration of osmoticum, be this indeed the event responsible for the development of the secretion pressure.

d) The Stimulus-response Relationship. In the general interpretation of the response to natural solid prey given in an earlier section, it was noted that the size of the secretion pool is related within limits to the size of the captured object. Glands in contact are quickly provoked to secrete, and then in turn others which become immersed in the advancing secretion pool (Fig. 16). The fact that the pool is limited according to the size of the capture suggests that the process is not relay-like; it can hardly be that the secretion of one gland is adequate itself to start the secretion of the next; otherwise, secretory activity would spread over an indefinite area. The most likely interpretation is that some of the products of digestion are carried outwards by the secretion pool, and that it is these that are responsible for the radial spread of activity. Should this explanation be correct, it remains enigmatic why the protein-containing discharge from one gland should

not be effective in provoking the secretion of another, but problems of this type are hardly unique in the general field of glandular physiology.

The autoradiographic evidence does show that digestion products spread within the secretion pool. Of course, the centrifugal movement does not depend wholly upon diffusion, since the secretion is actually flowing outwards from the vicinity of the original stimulus. Nevertheless there will be a radial concentration gradient, and the spread of the stimulus might be expected to cease at the point where the concentration fell below the threshold required to provoke secretion. The topography of the leaf, which becomes depressed near the prey (Batalin, 1877), will also tend to limit the spread of the area of secretion around small objects.

e) Resorption of Digestion Products. The autoradiographic study shows that resorption of the products of digestion begins very rapidly, and certainly while the secretion pool is still spreading. Radioactivity appears *within* the head cells of the sessile glands (Fig. 25), and very speedily in the vascular system marginal to the secretion pool (Figs. 19–22). While there is probably also some direct penetration into the leaf epidermal cells, the indications are that much of the digest is moved into the leaf through the very route taken by the secretion in passing out, namely, the sessile glands. It is hardly conceivable that such a two-way traffic could occur simultaneously, so it must be concluded that for each gland the phase of secretion gives place to one of resorption after quite a short interval. In the light of the interpretation of the mechanism of secretion given above, it can be seen that what would be required to bring about such a reversal would be the loss of semi-permeability by the plasma of the columella cell. The gland would then become a source rather than sink for fluid, which would be expected accordingly to move from the leaf surface into the xylem and be re-distributed to whatever areas of the leaf were developing water tensions at the time.

Nothing can yet be said about the relative rates of penetration of different molecules and ions into the *Pinguicula* leaf. However, since the sessile glands are concerned both with secretion and resorption, the process may very well be different from that described by Lüttge (1963, 1966b) in *Nepenthes*. The roof glands at the bottom of the *Nepenthes* pitcher are concerned with the controlled uptake of materials from the pitcher fluid, and this may not involve the passage into the leaf tissues of large volumes of liquid, as it undoubtedly does in *Pinguicula*.

A final point to be made concerns the speed of the digestive process in *Pinguicula*. Darwin's (1875) estimates of the timing of secretion have been abundantly verified in this study; there is no doubt that digestion begins within an hour or so of stimulation, and that the

products start to pass into the leaf almost immediately. This may be compared with the much more leisurely pace of digestion and resorption in *Dionoea*. Here, the complete cycle from closure of the trap to re-opening takes 7–10 days (Darwin, 1875), within which period secretion takes place, the prey is digested, and the products resorbed (Lüttge, 1963). Schwab *et al.* (1969) report that measurable amounts of proteolytic enzymes are present in the digestive fluid within 36 h of trap closure. How much earlier than this secretion actually starts has not been recorded, but these authors state that the first fine-structural changes are seen in the glands 1–2 days after closure, which seems to suggest that secretion does not begin until after 24 h or so.

The difference in the rate of digestion between *Pinguicula* and *Dionoea* may be related to the mode of enzyme storage. Enzymes are abundantly present in the spongy walls of the gland cells of *Pinguicula*, and in an active form, if the cytochemical results may be taken so to indicate. They are thus available for immediate discharge. Schwab *et al.* (1969) suggest tentatively that in *Dionoea* the enzymes are stored in the gland cell vacuoles, and "in this respect are similar to zymogen granules" On this basis, activation and release would require a different—and evidently more slowly acting—mechanism from that seen in *Pinguicula*.

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