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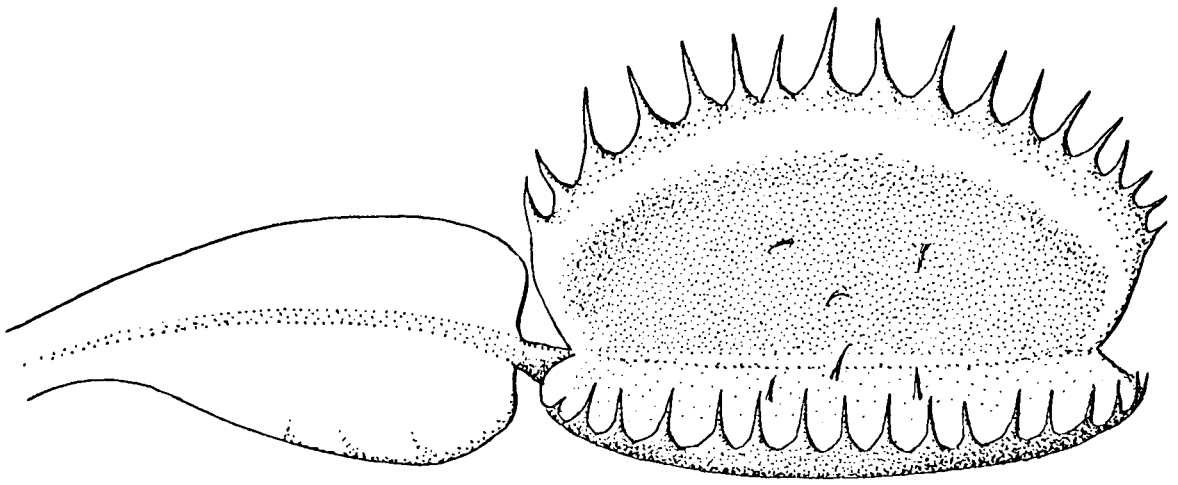
The Venus's Flytrap *Dionaea muscipula* Ellis

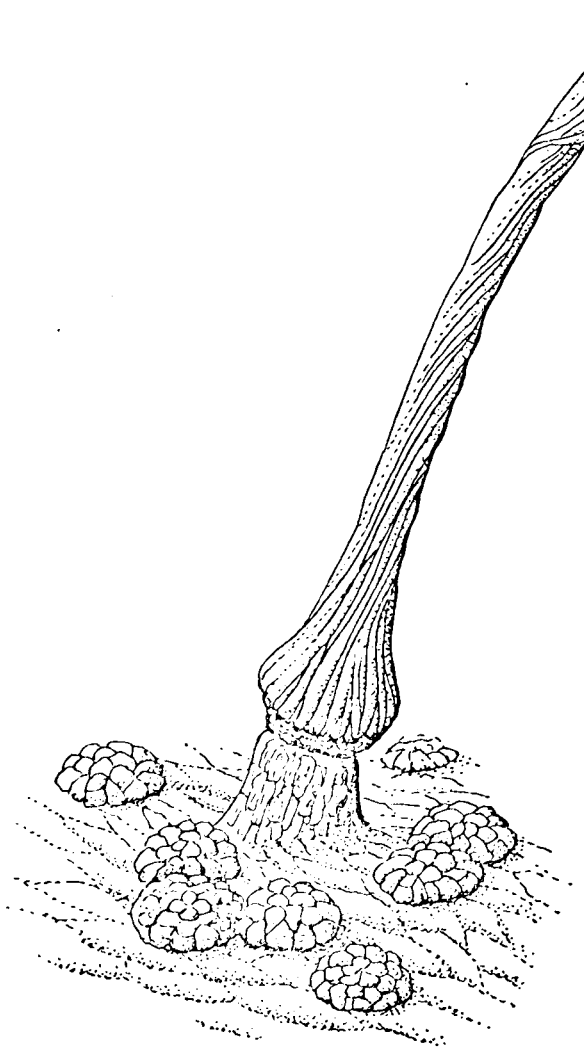
›p centre: a single leaf showing the lobed-trap.

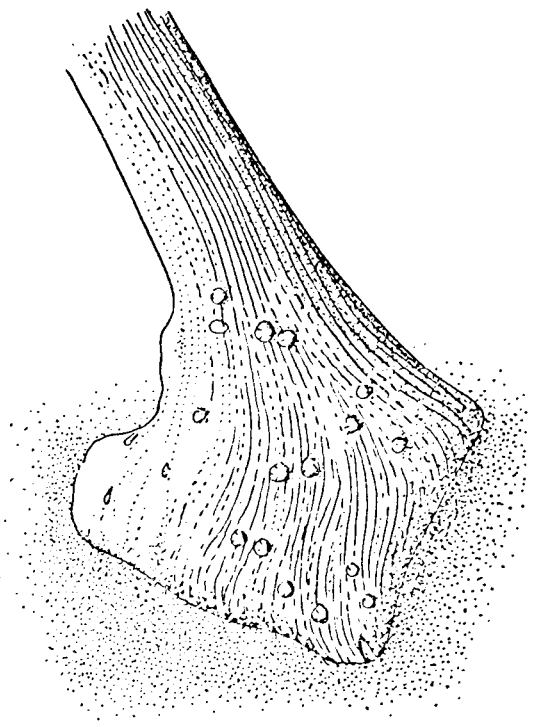
›wer left: a trigger hair, surrounded by digestive glands.

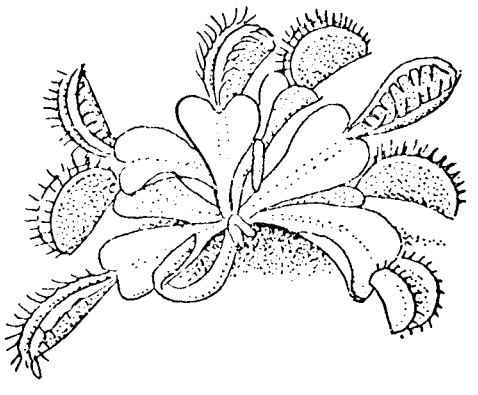
›per right: a portion of the lobe-margin showing the base of a marginal projection and some small non-digestive secretory glands.

wer right: the whole plant.









Rosemary Wise.

STUDIES

ON SECRETION AND ABSORPTION

IN *DIONAEA MUSCIPULA* ELLIS

"The special field of gland physiology
requires the integration of many experimental approaches"

Ulrich Lüttge (1971)

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Thesis presented for the degree of

Doctor of Philosophy

at the University of Oxford

by

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Pembroke College,
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Long Vacation, 1978

CORREGIA

PAGE	LINE	INCORRECT	CORRECT
v	7	these	them
ix	15	pores	pore
x	12	N,N,N,'N'	N,N,N',N'
19	17	mm	nm
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50	24	interval	internal
62	11	-Nitrophenyl	-nitrophenyl
64	11&12	Horseradish Peroxidase	horseradish peroxidase
66	4	Dimethyl	dimethyl
Fig. 2.1	1&9	ellution	elution
77	28	Fig. 3.27	Fig. 3.28
93	1	endodermis	epidermis
95	10	a gland	glands
	29	area	volume
96	27	hydrathodes	hydathodes
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3.53	9	74,400	109,200
130	24	3.9	3.95
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140	14	intercollated	intercalated
147	page no.	147	145 etc to end
Fig. 4.19	3	4.18:4.19:4.20	4.18:4.20:4.21
150	2	Jamison	Jamieson
153	5	with...	with two or...
164	6	enzymatic	enzymic
169	3	aetinomycin	actinomycin
	5&10	Morre	Morre
173	26	-nitrophenyl	-Nitrophenyl
174	5	4.95	3.95
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Fig. 6.2e	Plate		insert scale 0.1,0.2, 0.3 on ordinate
188	24	microscope	microprobe
193	3	7.10a	7.10b
	12	7.10b	7.10a
211	diagram:	r/p is width of plasmodesma visible and not total diameter	
Fig. 7.10	insert below 7.10b:	significance levels; inner face of OSC vs. outer face of ISC - NS all other interfaces - ***	
7.10	Plate (c)	add: OSC, ISC, SC to figure	
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253	1	Leucine	leucine
Fig. 2.2	(c)	33.4	3.4
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SOME OF THE WORK PRESENTED HERE HAS BEEN PUBLISHED IN THE FOLLOWING:

Juniper, B.E., Gilchrist, A.J., and Robins, R.J. (1977) Some features of secretory systems in plants. Histochem. J. 9: 659-680.

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ABSTRACT

The ultrastructure and physiology of the secretory glands of Dionaea muscipula Ellis (The Venus's Flytrap) have been examined in order to investigate the mechanism of obtaining nutrients practised by this plant.

The dynamic changes in the ultrastructure following stimulation have been examined. Particularly prominent features are a decrease in the size of the main cell vacuole and an increase in smaller vacuoles. Important changes are also observed in the distribution of ribosomes within the secretory cells. By the use of high resolution histochemical, cytochemical, and autoradiographic methods, it is shown that the secretory hydrolases are probably stored in a sub-compartment of the vacuole, the smooth endoplasmic reticulum and the cell walls. It is shown by quantification of the cell and by autoradiography that the dictyosomes do not appear to be involved in the discharge of secretion, but rather that this probably occurs both by direct fusion of the endoplasmic reticulum with the plasmalemma and by the migration of vesicles derived from the endoplasmic reticulum directly to the cell periphery.

Evidence is presented to show that, in addition to being released from a site of storage, some of the protein discharged is synthesized de novo during the cycle. This is suggested by the observed formation of numerous polysomes associated with the endoplasmic reticulum in the secretory cells and confirmed by radiolabelling techniques.

A preliminary analysis of the enzymology of the secretion is presented and it is tentatively suggested that there are several peptide hydrolase activities present, possibly including a serine proteinase and a carboxypeptidase. It is shown that the secretion can hydrolyse chitin and has peroxidase activity.

By following the absorption of chloride ions, it is shown that the plasmodesmata are important in the uptake of these ions. The overall architecture of the gland is considered in relation to the possible pathway of absorption of digestive products and it is suggested that the plasmo-desmata are probably the primary route for the movement of nutrients into the glands.

A model is put forward, based on these observations, for the way in which the gland conducts a bi-directional flow of material. It is hypothesized that, while absorption takes place via the symplast, secretory proteins are dis-charged directly into the apoplast and flushed from there by hydrostatic pressure generated by the inner secretory cells. Suggestions are made for how this might be achieved.

ABBREVIATIONS

APS	Ammonium persulphate
ATEE	N-Acetyl-L-tyrosine ethyl ester
ATP	Adenosine 5'-triphosphate (disodium salt)
BAME	α-N-Benzoyl-L-arginine methyl ester
BC	basal cell
bis	N,N'-Methylenebisacrylamide
BSA	bovine serum albumin (crystallized)
Butyl-PBD	2-(4'-tert-butylphenyl)-5-(4"-biphenylyl)-1,3,4-oxadiazole
C	cuticle
CBZ	benzyloxycarbonyl
Ch	condensed chromatin
Cr	cristae
CW	cell wall
1^{o}CW	primary cell wall
D	dictyosome
DMAB	4-Dimethylaminobenzaldehyde
DMSO	dimethyl sulphoxide
Dt	desmotubule
DV	dictyosomal vesicle
E	epidermal cell
Ec	endocuticle
EDTA	ethylenediaminetetraacetic acid (sodium salt)
ER	endoplasmic reticulum
GP	ground-plasm
IB	inclusion body
IC	intracisternal space

ISC	inner secretory cell
L	lomasome
LD	lipid droplet
LW	labyrinthine wall
M	mitochondrion
Mb	microbody
MB	membranous body
ML	middle lamella
MS	membrane-free space
MVB	multivesicular body
N	nucleus
NAG	N-Acetyl-D-glucosamine
NE	nuclear envelope
NL	nucleolus
NP	nuclear pores
NPE	nitrophenyl ester
NS	not significant
OSC	outer secretory cell
P	plastid
PAGE	polyacrylamide gel electrophoresis
PB	protein body
Pd	plasmodesmata
Pg	plastoglobuli
PM	plasma membrane
PMSF	phenylmethyl sulphonyl fluoride
POPOP	1,4-Di[2-(5 phenyloxazoyl)]benzene
PP	proplastid
PPO	2,5-Diphenyloxazole
pR	polysome
R	ribosome

rER	rough endoplasmic reticulum
SC	stalk cell
SDS	sodium lauryl sulphate
SE	subepidermal cell
SER	smooth endoplasmic reticulum
SG	starch grains
SW	secondary wall
T	tonoplast
TAME	N-α-Toluene-4-sulphonyl-L-arginine methyl ester
TC	tubular complex
TCA	trichloroacetic acid
Temed	N,N,N,'N'-Tetramethylethylenediamine
TER	total endoplasmic reticulum
Th	thylakoid
Tris	2-Amino-2-hydroxymethylpropane-1,3-diol
TV	transition vesicle
V	vacuole
V_c	clear vacuole
V_g	granular vacuole
V_l	large vacuole
V_s	small vacuole
Ve	vesicle
v/v	volume/volume
w/v	weight/volume
w/w	weight/weight

CHAPTER 1

INTRODUCTION

The Venus's flytrap has fascinated many eminent scientists ever since its first description by Arthur Dobbs in 1760. Described as "the great wonder of the vegetable kingdom" by its discoverer and as "miraculum naturae" by Linnaeus (Curtis, 1834), Charles Darwin considered it "the most wonderful plant in the world" (Jones, 1923). Its carnivory was first recognised by Ellis who described the capture of insects by this plant in a letter to Linnaeus dated about 1768 (Hooker, 1875). Furthermore, Ellis appreciated that the entrapping of insects was a deliberate act to obtain nutrients. Linnaeus, however, was unable to accept this (Linnaeus, 1771) and his authority over-rode all criticism until the detailed description by Curtis in 1834 (Curtis, 1834) of the capture and digestion of insects and spiders by plants growing in the wild.

Dionaea muscipula Ellis grows as a compact rosette of six to eight leaves, which lie on the ground (see Frontispiece). Each leaf has an obcordate 'footstalk' which terminates in the trap. This, which Ellis (1770) described as "a miniature form of rat-trap" and Curtis (1834) as "two upper eyelids joined at their bases", has two curvi-linear trapezoid half-lobes, each fringed with ribbed prongs. The abaxial surface of each lobe is densely covered with small secretory glands, coloured bright red (Fig. 1.1) by the cyanidin-3-glucoside contained within them (Di Gregorio and Di Palma, 1966). Each half-lobe is surmounted by three mechano-sensory trigger hairs (Fig. 1.1) which have been shown to be responsible for initially detecting the presence of prey and then for operating the closure mechanism.

The earlier experiments on the rapid movement of these traps are excellently summarized by Lloyd (1942). Burdon-Sanderson (1873) demonstrated that flexing the trigger-hair induced a propagated action potential over the surface of the leaf. One flexure is, under normal conditions of temperature and humidity, insufficient to cause trap closure (Macfarlane, 1892), a second within a short time of the first usually being needed. Brown (1916) showed that the longer the time between flexures then the greater the number required to cause closure but that two applied to any of the hairs within 30 s of one another were normally sufficient. Brown and Sharp (1910) demonstrated that the site of detection of flexure lay at the base of the trigger hair since if the portion above the swelling (see Frontispiece) was removed it was still sensitive to mechanical stimulation. Jacobson (1965) confirmed that the two specialized layers of sensory cells at this point (Williams and Mozingo, 1971) are the site of initiation of the action potential, polarity being induced in them by mechanical pressure (Benolken and Jacobson, 1970).

Jacobson (1965) considers that there is some form of a 'memory' for these action potentials and that this 'memory' records the amplitude of the action potentials, rather than the number of stimuli received. As the action potential propagated from one trigger hair sensitizes the whole leaf surface such that a second flexure from any one of the six trigger hairs is equally effective in causing closure, the 'memory' may be a polarization of the cells over the whole epidermal layer. The evidence provided by Brown (1916), that the 'memory' decays at a rate such that the number of

flexures required to close the trap is approximately equal to the interval of time in minutes between each flexure, supports this idea.

Lea (1976) proposed that trap closure operates by a chemical system involving the stimulation of phospholipase D and the production of a "muscle contracting substance" which he tentatively identifies as lysophosphatidic acid. This seems highly implausible and is incompatible with the observed rate at which the trap responds. Benolken and Jacobson (1970) state that the trap may complete its closure to the 'narrowing' position (Fig. 1.1) within 100 ms of the last stimulus. As physical movement of the trap occupies most of this time, it is difficult to see how the transmission of the signal to close could be by a chemical messenger.

The following proposals may therefore be put forward as a model for the operation of the rapid-closure mechanism:

a) There is a minimum level of polarity within the 'memory' (possibly the epidermal cells) required to trigger the closure system;

b) This polarity is induced by action potentials propagated by flexure of the trigger hairs;

c) The minimum required level of polarity is slightly greater than may be induced by a single action potential but considerably less than the sum of two action potentials;

d) The 'memory' has a finite lifetime, decaying at such a rate that a second action potential must follow the first within 30 s for the remaining amplitude to be sufficient to

fire the trap.

e) The 'memory' decays in an exponential fashion so that numerous mechanical stimuli with long time intervals between them are able to cause trap closure.

In the unstimulated state, the trap is set with the sides of the lobe open at an angle of $30 - 80^{o}$ (Fig. 1.1), with the adaxial surface being slightly convex and the abaxial surface correspondingly concave. Under these conditions it appears to be in a state of tension and Burdon-Sanderson (1882) suggested that the loss of this tension from the cells of the leaf tissue may cause the trap to close. Brown (1916) found negligible changes in the distances between dots of black ink applied to the adaxial leaf surface while the distance between those on the abaxial face increases by between 1.8 and 30.0%. This disagrees with the earlier suggestion of Darwin (1875) that the motion involves a shrinkage of the adaxial cells as well as an expansion of the abaxial. The midrib is not involved in the movement, as suggested by Ziegenspeck (1925), but rather the tension is apparently held in the parenchyme tissue of the trap (Lloyd, 1942).

How this closure is induced by the stimulation of the trigger hairs is unknown but it is probable in view of the evidence cited above that the stimulus is transferred from its site of perception to its site of action by an electrical mechanism.

On closure, the trap initially adopts the 'closing' position (Ashida, 1934) as seen in Fig. 1.1, suggested by Jones (1923) to allow prey below a minimum size to escape.

This will be considered further in Chapter 8. Darwin (1875) found that when he stimulated traps to close over indigestible material such as sand, glass, wood or paper, no further closure occurred and they subsequently re-opened within 24 h. Numerous other workers have confirmed this. Darwin (1875) further found, however, that if digestible matter such as roast beef or egg albumin was present then the traps continued to close slowly over a period of several hours, finally adopting a position with the edges tightly appressed. This slow-closure response could be achieved without the previous rapid-closure if pieces of meat were carefully placed on the leaf so as not to disturb the trigger hairs (Darwin, 1875). From this it appears that the slow-closure response can be induced chemically. This was confirmed by Lichtner and Williams (1977), who found that ions such as sodium and ammonium were most effective at causing the slow-closure response, producing above 50% 'narrowing' at concentrations in excess of 2 mM. Glycine and L-lysine were also effective but they found salt-free ovalbumin to be much less effective and therefore suggested that the closure seen by Darwin (1875) may have been due to salts within his ovalbumin preparation.

Hence it appears that the trap initially closes within 100 ms to the 'closing' position, in response to a mechanosensory/electrical stimulation, and subsequently narrows in response to chemical stimulation.

In the same experiments, as mentioned earlier, Darwin (1875) found that stimulation of digestive fluid only took place in the presence of moist organic matter and that dry

meat or inorganic matter failed to produce secretion. I confirmed (Robins, 1975; 1976) that secretion is produced in response to nitrogenous molecules and further found that the volume and protein content of the secretion depends on the nitrogenous molecule exhibited and that these quantities appear to vary semi-independently. On the basis of these results, I proposed that the release of fluid and protein may be under separate controls and this may have been confirmed by Lichtner and Williams (1977), if, as may be the case for reasons suggested later, the fluid secreted in their experiments lacks digestive activity.

To summarize the closure system, then, there is an initial rapid closure in response to flexure of the trigger hairs and mediated by an electrical action potential, followed by a slow closure in response to chemical stimulation. If the former is activated without the latter stimulus then being applied, the trap re-opens. If the latter stimulus is applied (even without the former) the trap adopts the 'narrowing' position and starts secreting digestive fluid.

The mechanism by which digestive fluid is released from the glands and the hydrolytic activity of that fluid has received comparatively little attention. Lloyd (1942) summarizes the earlier experiments, such as those of Balfour (1875) and Darwin (1875), that showed the secreted fluid to be able to digest protein and absorb the products. Since 1942, however, only Lüttge (1964a) and Scala et al (1969) have made any further contribution to the enzymology of the secretion, demonstrating several peptide hydrolase activities

as well as esterase, glycosidase and anhydrase activities. The details of this are discussed in Chapter 6.

The structural side of the secretory mechanism has, similarly, received very little attention with the only sub-structural examination of the glands being that of Scala and co-workers (Scala et al, 1968a, b; Schwab et al, 1969) while Mozingo et al (1970) examined the surface features of the traps in the scanning electron microscope. On the basis of their qualitative observations of the dynamic changes in the ultrastructure of the gland cells, Schwab et al (1969) put forward a preliminary scheme for the structure/function relationships of the secretory gland.

A detailed examination of the mechanism by which digestive fluid is released from the glands and the hydrolytic activity of that fluid is the subject of this thesis.

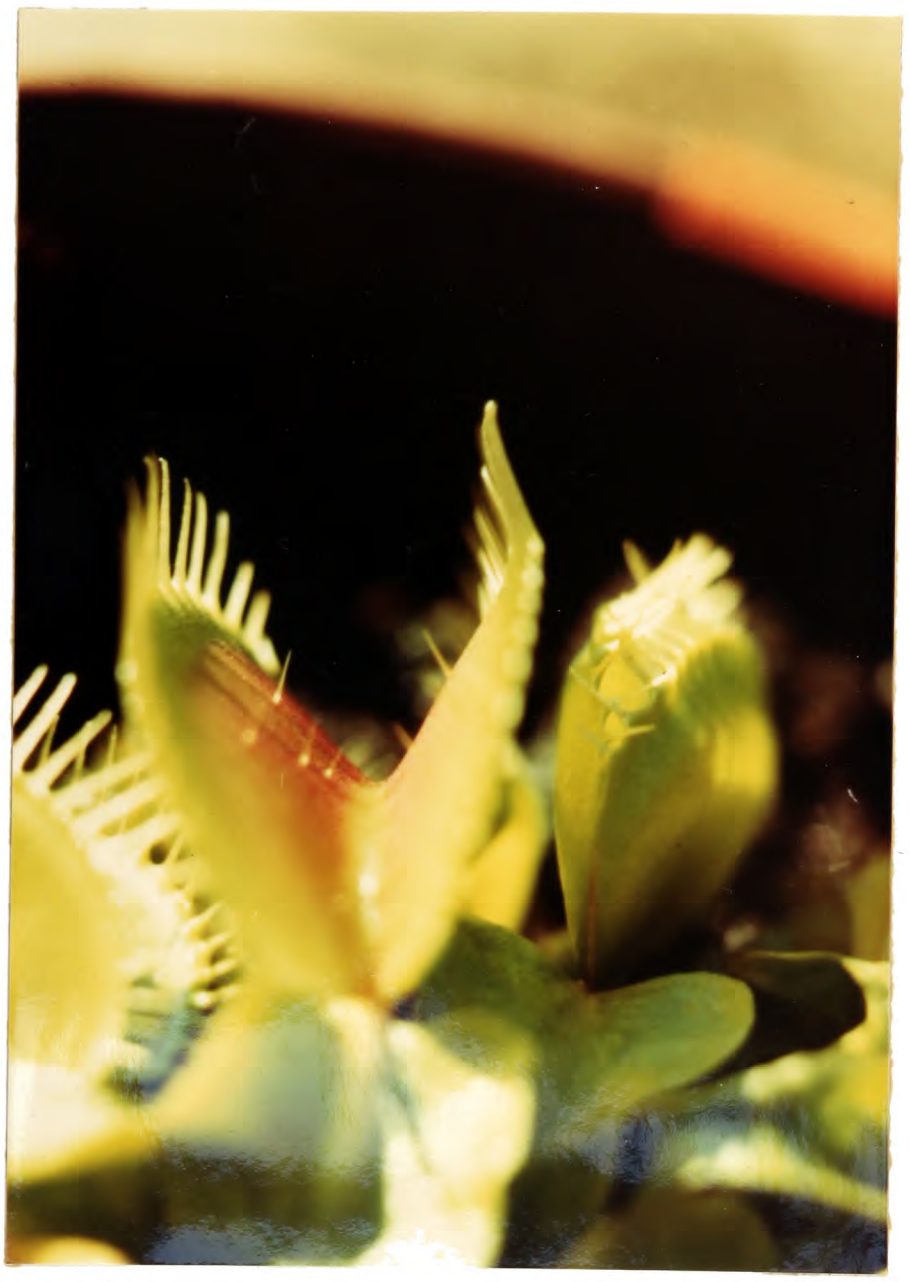


Figure 1.1 Showing left, an open unstimulated trap, and right, a trap in the 'closing' position. Note the three trigger hairs on both parts of the lobe and the bright red digestive glands. x 2.

CHAPTER 2

MATERIALS AND METHODS

I MATERIALS

1) Biological Material

2) Reagents

II MICROSCOPY

1) Fixation

2) Dehydration and Embedding

3) Ultrathin Sectioning

4) Section Staining

5) Electron Microscopy

6) Autoradiography

7) Cytochemistry

8) Thin-section Histochemistry

9) Scanning Electron Microscopy

10) High-voltage Electron Microscopy

11) Light Microscopy

12) Quantitative Image Analysis

III FEEDING AND COLLECTING METHODS

1) Stimulating the Glands

2) Collecting the Secretion

3) Estimation of the Area of the Traps

4) Radiolabelling of the Secreted Protein

5) Treatment with Inhibitors of Metabolic Processes

6) Density Labelling of the Secreted Protein

IV SEPARATION METHODS

1) Gel Fractionation
2) Polyacrylamide Gel Electrophoresis
3) Polyacrylamide Gel Isoelectric Focussing
4) Isopyknic Ultracentrifugation
5) Ion-exchange
6) Dialysis

V ASSAY METHODS

1) Protein
2) Proteolytic Activity
3) Other Enzymes
4) Radioactivity

I MATERIALS

1) Biological Material

Dionaea muscipula Ellis were supplied as seedlings by the Royal Botanic Gardens, Kew, in July 1974 and maintained in a shaded greenhouse at the Botanic Gardens, Oxford, during the remainder of the summer. Plants were left uncovered, so as to allow normal feeding to occur, and watered with rain-water twice-daily. Despite these uniform conditions the plants showed considerable variation in the amount of growth and a number died.

During the winter months Dionaea grows very slowly under greenhouse conditions. In October the 14 remaining plants were transferred to an environment-controlled growth cabinet in the Botany School, Oxford, and maintained at 25 ± 1°C under 10,000 lx illumination with a 12 h day. After two weeks in the cabinet, during which plants were fed twice with a few Drosophilia virilis per lobe, they were re-potted into new compost of acidic leaf mould/Sphagnum moss/ peat (1:1:1) top-dressed with Sphagnum moss. Young corms were split from the parent plants.

The design of the cabinet makes the conditions very desiccating and plants were normally watered twice-daily with rain water. In addition the pots stood in zinc trays containing washed gravel and plenty of water to ensure a high humidity in the immediate vicinity of the plants.

Initially plants grew vigorously and a number flowered,

but by June 1975 they began to regress and therefore were planted out into a bed of the same compost in a shaded greenhouse on the roof of the Botany School. Once again vigorous growth ensued, with a number of plants flowering.

In September 1975, at the start of the experimental work reported here, all the plants were removed from the bed and vegetative progeny once again divided from the parent corms. Those plants of insufficient size for experimental work were returned to the bed to over-winter, while the larger individuals were potted in fresh compost and transferred to the growth cabinet, which was set on a 16 h day (25 ± 1°C : 10 000 lx).

This cycle of conditions was maintained during the experimental period with plants moved to the greenhouse in late March or early April and back to the growth cabinet in September or October. Each time they were re-planted the vegetative progeny were divided from the parents and planted separately. It was found that by using this combination of conditions material was available throughout the year. In February and March, when plants in the growth cabinet had started to regress, then those that had over-wintered in the greenhouse had grown sufficiently for material to be obtained from these.

2) Reagents

Unless stated to the contrary in the text, the reagents used were supplied by the British Drug Houses Ltd., Poole

(BDH), or by the Sigma Chemical Company Ltd., Poole (SCC), and were of the highest grade commercially available. Specialist materials for electron microscopy were purchased from TAAB Laboratories Ltd., Reading, or EMscope Laboratories Ltd., London, except for the osmium tetroxide which was from BDH. The Bactopeptone 'Difco' preparation was from the Difco Labs., Detroit, U.S.A.

Radiolabelled compounds were obtained from the Radiochemical Centre, Amersham, and were supplied as follows: L-[4,5-^{3}H]-leucine as a 1.0 mCi ml^{-1} solution in 2%(v/v) ethanol; L-[U-^{14}C]-leucine as a 50 µCi ml^{-1} solution in 2%(v/v) ethanol; [U-^{14}C]-protein as 100 µCi of freeze-dried powder.

Dyestuffs were supplied by George T. Gurr Ltd., London; material for gel filtration was purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden; Ampholines were bought from LKB-Produkter AB, Bromma, Sweden.

Lipoluma, Liposolve and Lipogel are the registered names of scintillants prepared by Lumac Systems AG, Basel, Switzerland, and distributed by Norlab Ltd., Macclesfield.

II MICROSCOPY

1) Fixation

The trap selected for examination was cut through the petiole and placed immediately into a petri-dish containing fixative solution. The two lobes were separated by a cut along the midrib and the non-glandular tissue trimmed from the area to be fixed. This was cut to give pieces approximately 0.5 - 1 mm wide by 2 - 3 mm long, the long axis being perpendicular to the midrib. For cytochemical fixations (see 2:II:7) the pieces used were very much smaller, so as to improve the penetration of substrates into the tissue (Sexton et al, 1971).

1. Potassium Permanganate. Treatment of tissue with a 2%(w/v) potassium permanganate aqueous solution for 75 min at 4°C was tried but found to be unsatisfactory, leaving little structural integrity within the gland-cells. It was, therefore, not used for routine fixation.

2. Glutaraldehyde - Osmium tetroxide. Gland material is notoriously difficult to prepare and a number of schemes were used. Material was:

(i) fixed for 4 h in 2.5%(w/v) glutaraldehyde in 20 mM-2, 4, 6,-Collidine buffer containing 200 mM-sucrose (pH 7.4 : acetic acid) at room temperature and postfixed for 1 h in 1%(w/v) osmium tetroxide in the same buffer at room temperature.

(ii) fixed for 4 - 16 h in 3%(w/v) glutaraldehyde in 100 mM-sodium cacodylate buffer (pH 7.2 : acetic acid) under suction from a water vacuum pump at room temperature and postfixed for 1 h in 1%(w/v) osmium tetroxide in the same

buffer at room temperature and pressure.

(iii) fixed for 2 - 6 h in 3% (w/v) glutaraldehyde in 100 mM-sodium cacodylate buffer (pH7.2 : acetic acid) at 4°C and postfixed for 2 - 6 h in 1% (w/v) osmium tetroxide in the same buffer at room temperature.

Scheme (iii) proved the most satisfactory and was used for routine preparation.

Material was cut as described above in cold 3% (w/v) glutaraldehyde in 100 mM-sodium cacodylate buffer and fixed for 2 - 6 h in this solution at 4°C in a vial fitted with a plunger to hold the pieces of tissue below the surface. It was then washed in cold buffer with 4 - 6 changes at 10 min intervals and 2 changes at 20 min intervals. During the last wash vials were removed from the cold room (4°C) and left on the bench to allow the temperature to rise slowly to laboratory conditions. The material was then transferred to sealed vials and postfixed for 2 - 6 h in 1% (w/v) osmium tetroxide solution in buffer at room temperature, washed in buffer with one immediate change and 2 - 4 changes at 10 min intervals and left overnight at 4°C.

Despite considerable care being taken to standardize the conditions of fixation some variation in the overall appearance of the cells occurred, probably due to the metabolic state of the cells at the time of fixation. It was, therefore, necessary to include an untreated gland as a control in any fixation which involved a comparison of one treatment with another. Unless stated to the contrary, all material illustrated was prepared using the scheme detailed above.

2) Dehydration and Embedding

Fixed and osmicated material that had been left overnight at 4°C was left 20 min in the laboratory to allow temperature equilibration and then washed once, for 10 min in fresh buffer at room temperature. Dehydration was performed using a graded series of ethanols, spending consecutive periods of 10 min in each of 25, 50, 70, and 90%(v/v) ethanol solutions, followed by 10 min in each of 2 changes of absolute ethanol. In later preparations an initial 10%(v/v) ethanol step was included.

At first material was embedded in Epikote-812 (Luft, 1961) but severe problems with poor penetration were encountered and subsequently Spurr's low-viscosity resin (Spurr, 1969) was used, which proved much more satisfactory.

1. Epikote-812. Dehydrated material was washed through a graded series of acetone/ethanol mixtures, spending 10 min in each of 1:3, 1:1, and 3:1, followed by 2 changes in 100% dry acetone.

Resin was prepared volumetrically by mixing:

Component	Quantity
Epon-812	16 ml
DDSA (dodecenylsuccinic anhydride)	10 ml
MNA (methyl endomethylene tetrahydrophthalic anhydride)	9 ml
DMP-30 (2, 4, 6-Tri(dimethylaminomethyl)phenol)	2 drops

An equal volume of freshly prepared resin was added to the material in acetone, mixed thoroughly, and left to stand open overnight in the fume cupboard with air being drawn

over the vials. The following morning material was moved into fresh resin and left under suction from a water vacuum-pump for 3 h, after which the resin was again changed and left a further 5 h *in vacuo*.

Gelatin capsules (Parke-Davis & Co., Pontypool), held in aluminium blocks, were filled with freshly made resin and individual pieces of tissue placed on the surface of the fluid. Resin was set by incubating it at 60°C for about 48 h. During the initial stages, as the resin warms up and becomes less viscous, the pieces of tissue slowly sink to the bottoms of the capsules and are thus embedded in pure, fresh resin. After incubation, tissue pieces were cut from the capsules and orientated as required (see 2:II:3).

2. Spurr's low-viscosity resin. Because this resin is miscible with ethanol no transfer to acetone was required.

Resin was prepared gravimetrically by mixing:

Component	Quantity	
	Normal formula	Hard formula
ERL-4206 (vinyl cyclohexene dioxide)	10.0 g	10.0 g
D.E.R.736 (diglycidyl ether of polypropylene glycol)	6.0 g	4.0 g
NSA (nonenyl succinic anhydride)	26.0 g	26.0 g
S-1 (dimethylaminoethanol)	0.4 g	0.4 g

The formulation was thoroughly mixed after the addition of each ingredient. When homogeneous the resin was degassed for about 30 min under suction from a water vacuum pump. The same mix was used throughout the embedding schedule.

Material was placed in a small volume of absolute

alcohol and an equal volume of resin added and mixed in. The solution was gently agitated by rotating the vial on a wheel (about 20^{o} slope: 9 rev min^{-1}). After 30 min a further portion of resin (equal to the initial volume used) was added and agitation continued. The addition of resin was repeated twice more at 30 min intervals. The vials were then drained, fresh resin added and agitation continued for 3 - 4 h. Once again the vials were drained, fresh resin added and the vials left open overnight in the fume cupboard with air drawn over their tops. Pieces of tissue were cast in fresh resin by incubating for 8 - 10 h at $70^{o}C$.

Either individual pieces of tissue were embedded in oven-dried gelatin capsules using the procedure described above, or a number of pieces were cast into a single block using the lower part of a disposable plastic beaker as a mould. The choice of method was determined by the number of different treatments being handled together and the number of samples required from each treatment.

In one or two preparations the hard-setting formulation was used when a batch of tissue was presenting particularly bad penetration problems. Also, when this was the case, the tissue was soaked for an extra day in resin before being cast. Despite this there were occasions when the embedding was very poor and material had to be discarded.

3) Ultrathin Sectioning

Embedded material nearly always had to be re-orientated before being sectioned. For this it was cut from the cast

and set with araldite resin (CIBA-GEIGY Plastics and Adhesives Co., Cambridge) on a short length of wooden dowel. Usually the material was orientated so that the block face formed a section parallel to the midrib of the lobe and the ultrathin sections were cut through the glands and underlying tissue perpendicular to the leaf surface. This plane of section was found to be the most satisfactory for interpreting the gland's ultrastructure.

All sections were cut on a Reichert OMU2 ultramicrotome and floated onto either pure distilled water or distilled water with a few drops of acetone added per 100 ml. Block faces were trimmed to a trapezium of about 1-2 x 0.5-1 mm.

1. Glass knives. These were made on an LKB 7801B knife-maker using 25 mm width glass, broken to give a cutting edge of 45°. Troughs were made from either Scotch tape or LKB Trufs sealed with soft wax. With these knives, set at 5 - 7°, good ribbons of gold-silver and silver sections (90 - 120 mm) could be cut and sections showed very little folding or chatter. When this did occur then it was removed by treating with chloroform vapour. The cutting edge rapidly became blunt but one knife would normally cut 3 or 4 grids.

2. Diamond knife. Even taking the greatest care sections cut on a Ge-Fi-Ri diamond knife, set at 3 - 5°, tended to be chattered. Material embedded in Epikote-812 cut better than material in Spurr's resin but for routine use glass was found to give a more satisfactory cutting edge. Sections were cut on diamond where a large number of serial sections was needed, notably for thin-section histochemistry.

For normal viewing, sections were picked up from below

onto the dull side of 200 mesh copper grids, immediately pre-washed in acetone. For autoradiography and enzymatic digestion, uncoated 200 mesh nickel grids were used. When serial sections were to be examined, or mosaics to be made, these were picked up onto 100 mesh copper grids coated with Formvar film.

Formvar film was prepared from 0.25%(w/v) polyvinyl formal (Shawinigan Ltd., London) in chloroform. Finger-greased glass slides were dipped and allowed to drain vertically for a few minutes until dry. The edges were scored and the film floated onto a bath of distilled water. Grids were dropped onto the film, dull-side down, and the whole preparation lifted with a filter paper. Coated grids were always left overnight before use and kept satisfactorily for 1 - 2 months. Wrinkles, such as discussed by Cox (1971), were not found to be a problem.

4) Section Staining

For routine examination sections were either stained with alkaline lead II citrate solution (Reynolds, 1963) or double stained with saturated aqueous uranyl acetate solution followed by the alkaline lead II citrate treatment.

1. Lead II citrate. Sections were stained by floating section-side down on droplets of alkaline lead II citrate solution in a closed waxed petri-dish, in the presence of pellets of sodium hydroxide to prevent contamination by crystals of lead II carbonate. Staining was performed for 10 - 20 min (normally 15 min) after which grids were washed thoroughly with distilled water.

2. Uranyl acetate - lead II citrate. Sections were stained by floating grids section-side down on droplets of saturated aqueous uranyl acetate solution on a wax block for 20 min. Staining was followed by 3 washes on droplets of water and 10 min staining with alkaline lead II citrate, as described above.

It is often difficult to obtain good contrast in Spurr's resin and if the contrast was poor material was normally discarded. With some of the blocks fixed for autoradiography however (see 2:II:6), it was impractical in the time available to prepare more material. The method of Bray and Wagenaar (1978), using a double staining technique with potassium permanganate - lead II citrate, was found radically to enhance the contrast and render this material satisfactory for use.

3. Potassium permanganate - lead II citrate. Grids were clamped section-side up in locking forceps and a drop of 1% (w/v) potassium permanganate aqueous solution applied to the upper surface. Staining was conducted for exactly 4 min after which grids were thoroughly washed with distilled water and stained in alkaline lead II citrate for 10 min, as described above.

The potassium permanganate solution need not be freshly made but the liquid used must be withdrawn from the body of the stock solution immediately prior to use. With this precaution, and by ensuring that the grids were thoroughly washed, negligible contamination of sections occurred.

It was found advisable to let sections dry for a few hours, preferably overnight, before viewing. If viewed immediately after staining they tended to be more fragile under the electron beam.

5) Electron microscopy

Sections were viewed on an AEI 801 electron microscope in the Botany School, Oxford, at an accelerating voltage of 60 kV. For normal viewing a 100 µm aperture was used but, because of the low contrast, it was often found necessary to use 50 µm and 25 µm apertures. The microscope is fitted with a nitrogen-filled decontaminator system.

Micrographs were taken on Ilford plates electron microscope type EM5 (medium contrast); Ilford film SP 332 (medium contrast) used as both cut film and 70 mm roll film; and Kodak electron microscope film 4489 (high contrast) used as cut film. All film types were developed under Ilford type F904 safelights in Ilford PQ Universal developer (diluted 1 + 9) for 4 min, washed in an acidic stop-bath, fixed 2 - 5 min in Ilford Hypam rapid fixer (diluted 1 + 4), and washed in running water for about 30 min.

Negatives were printed, using a Leitz Focomat IIC enlarger, onto Kodak Bromide photographic papers and Ilford YR rapid print papers. Kodak papers were developed in PQ Universal developer (diluted 1 + 9) for 2 min, washed in an acidic stop-bath, and fixed in Hypam fixative (diluted 1 + 4) for at least 3 min. Ilford YR papers were developed in a Kodak Auto-processor model Q14 using Ilford Ilfoprint 1A-11

activator and Ilford Ilfoprint IS-21 stabiliser. These prints were permanently fixed in Hypam, as above. After washing, prints were glazed to give a gloss finish.

Photographs used in this thesis were printed on Kodak Bromide papers, mounted, and each plate rephotographed onto 12.1 x 16.5 cm sheets of Ilford FP4 film, and reprinted onto Kodak Bromide paper. Magnifications were measured directly from the negatives.

6) Autoradiography

The aim of these experiments was to grow plants on a radioactive source of amino acid for a sufficiently long period so that the secretory proteins of the new leaves, which developed during the feeding period, would be radioactively labelled. They could then potentially be localized within the gland cells and the pathway of secretion followed by tracking the redistribution of silver grains in gland cells as a function of time after stimulation. It was also anticipated that an autoradiographic examination of glands during their development might help in establishing the mechanism used for storing the secretory proteins.

1. Preparation of material. Healthy plants, growing in pots in the greenhouse, were fed daily through the leaves with 200 µl per plant of a stimulating solution of 4% (w/v) bactopeptone (see 2:III:1) containing 5 µCi ml^{-1} of L-[4,5-^{3}H]-leucine. During the feeding period new leaves were tagged as they developed from the centres of the rosettes to ensure that the leaves used in the experiments had never been fed a radioactive solution.

After 14 days leaves at various stages of development were excised and prepared for electron microscopy. Sections of material prepared from mature traps were cut, mounted on nickel grids and stained with uranyl acetate and alkaline lead II citrate. In the first batch of sections prepared, half the grids from each block were given a thin protective coat (approximately 10 - 20 nm) of evaporated carbon in an Edwards Coating Unit model 12E6/599 before emulsion was applied. The presence of the film, however, did not seem to affect either the contrast of the sections after development or the grain density and was therefore not used in subsequent preparations.

The fixative and dehydration solutions were retained and the radioactivity present measured by liquid scintillation counting (see 2:V:4) to verify that some label was present in these leaves. Fixative solutions contained some pigment and were therefore decolorized with an equal volume of 30% (w/v) hydrogen peroxide solution overnight before scintillant was added. Alcoholic solutions did not need decolorising.

After a further 16 days, during which daily feeding was continued as before, a number of previously unfed mature traps, which had developed during the radiolabel-feeding period, were stimulated to secrete (see 2:III:1) with non-radioactive stimulating solution. These were harvested at 2, 4, 6 and 8 days after stimulation and prepared for electron microscopy. Despite following the standard preparation scheme (iii), the contrast was very low in this material and had to be enhanced with the potassium permanganate - lead II citrate method described previously.

2. Preparation of Autoradiographs. This was conducted using a slight modification of the film-loop technique described by Caro et al (1962), in which the grids were mounted on perspex stubs.

Perspex rod (5 mm diameter) was cut into lengths of approximately 10 mm and the ends filed smooth. These stubs were mounted with double-sided Sellotape onto glass microscope slides, 3 per slide. Two narrow strips of double-sided Sellotape were stuck to the top of the stub, spaced just under 3 mm apart. One grid was placed, section-side up onto each stub and held in place by its edges just coming into contact with the Sellotape. Slides were labelled in pencil on white adhesive tape and stored in light-proof boxes, with silica gel.

Emulsion was prepared under an Ilford F904 safelight using the method described in Caro et al (1962) except that only 15 ml of water was added to the 10 g of emulsion. Film was formed on an oval platinum wire loop of axial dimensions 20 x 30 mm and gelling was followed by watching the interference colours in the film under the safelight. Once a large enough area of a uniform gold colour had formed this was looped over the stub, ensuring that the golden area fell over the grid. The loop was cleaned each time in warm water.

Emulsion would normally remain workable for 15 - 20 min after which it could be satisfactorily reheated and cooled 3 or 4 times without any apparent effect on the stability of the emulsion. The period during which the emulsion was at a workable consistency, however, decreased after each reheating.

One batch was found to be sufficient to prepare 60 grids.

The boxes were sealed with black adhesive tape, sealed into a double light-proof plastic bag, and left to expose in the cold room at 4°C. Grids were removed and developed after 3 - 6 months exposure.

Autoradiographs were developed in Kodak D-19 developer for 2 min at room temperature, washed for 10 s in a 1% (v/v) acetic acid solution, fixed for 2 - 3 min in a 20% (w/v) sodium thiosulphate solution, and finally washed in 3 changes of distilled water. Processing was conducted on wax blocks onto which droplets of the processing solutions were syringed through millipore filters immediately prior to use. Grids were floated emulsion-side down onto the droplets. After drying they were viewed on the electron microscope without further treatment.

The distribution of silver grains was recorded directly from the electron microscope using high power magnification to assess the underlying organelles.

7) Cytochemistry

1. Iron II sulphate for tannins. Unstimulated traps were cut and fixed for 4 h as in scheme (ii). After washing, material was divided into three portions. One received no further treatment, one was postfixed with 1% (w/v) osmium tetroxide for 1 h, and one was treated with fresh 10% (w/v) iron II sulphate solution for 48 h at 4°C, with several changes of solution. All treatments were prepared for electron microscopy embedded in Epikote resin. Sections were

viewed unstained or treated with alkaline lead II citrate only.

2. Gomori reaction for phosphatases. The methods used were the modified Gomori reactions of Hall (1971) and Hall and Butt (1968). Material was cut, fixed for 2 h as in scheme (iii), thoroughly washed, and incubated at 25°C in a shaking water-bath in substrate medium (see below) for either acid β-glycerophosphatase or ATPase activity. In both cases controls were conducted using tissue boiled for 15 min and incubated in full medium and ordinary tissue incubated in medium without substrate. Pieces of tissue were withdrawn at various times and washed rapidly in several changes of buffer. Some pieces from each sample were post-fixed with buffered 1%(w/v) osmium tetroxide (scheme (iii)). All samples were prepared for electron microscopy embedded in Spurr's resin.

Media:

i) Acid β-glycerophosphatase. Freshly prepared: (a) 10 ml of 100 mM-sodium β-glycerophosphate solution (315 mg) adjusted to pH5.5 with acetic acid; (b) 100 ml of 4.2 mM-lead II nitrate solution (120 mg) in 50 mM-sodium acetate buffer (pH5.5). Immediately before use 5 ml of (a) was mixed with 50 ml (b) and filtered. A flocculent precipitate appeared after prolonged incubation. The remainder of (b) served for the control.

ii) ATPase. A freshly prepared solution (100 ml) containing 2.0 mM-ATP (125 mg), 2.0 mM-copper II acetate (40 mg) and 3.6 mM-lead II nitrate (119 mg) in 50 mM-Tris/hydrochloric acid buffer (pH7.0). A pale blue finely-divided

precipitate may appear during incubation. For the control this solution is made without ATP present.

3. Mercury II Chloride-bromophenol blue for protein. This stain was adapted from light microscopy (Johansen, 1940) to localize protein in the electron microscope.

Material was prepared, fixed in glutaraldehyde as in scheme (iii), washed and incubated in mercury II chloride-bromophenol blue solution for 30 - 120 min at room temperature. It was then rinsed in fresh water, and immersed in 0.5%(v/v) acetic acid for 30 min at room temperature with occasional shaking. Following a further wash it was immersed in 100 mM-sodium citrate buffer (pH6.5) for 5 min before being rinsed and left overnight at 4^{o}C in the dark in 100 mM-sodium cacodylate buffer (pH7.2). Each sample of material was divided into 2 portions, one of which was treated with 1%(w/v) osmium tetroxide in 100 mM-sodium cacodylate buffer (pH7.2) for 2 h at room temperature. Finally it was washed, dehydrated and embedded in Spurr's resin.

Medium: 2.5 g mercury II chloride was dissolved in 25 ml 95%(v/v) ethanol; 25 mg bromophenol blue was added and mixed thoroughly.

4. Silver I chloride precipitation. The method used by, among others, Harvey et al (1976) and Lüttge and Ziegler (1967) to localize endogenous chloride in plant cells by the precipitation of insoluble silver I chloride, was modified so as to examine the inward flux of exogenous chloride into the glands.

(i) Long time-course. Mature leaves (2) from one plant were fed a stimulating solution of 2%(w/v) bactopeptone (see

2:III:1) containing 100 mM-sodium chloride 3 times per day for 2 days. On the third day they were excised and the pieces were treated as follows. Unstimulated leaves were used as controls.

a) fixed for 2 h in 0.5%(w/v) osmium tetroxide plus 0.25% (w/v) silver I nitrate in 50 mM-sodium cacodylate buffer (pH7.2) under vacuum in an ice bath;

b) fixed for 2 h in 3%(w/v) glutaraldehyde plus 0.25%(w/v) silver I nitrate in 50 mM-sodium cacodylate buffer (pH7.2) under vacuum at room temperature;

c) fixed for 3 h in 3%(w/v) glutaraldehyde in 50 mM-sodium cacodylate buffer (pH7.2) under vacuum at room temperature.

(ii) Short time-course. Four mature leaves were stimulated with 4%(w/v) bactopeptone for 3 days. On the fourth day they were excised, cut down the midrib and the lobes placed with the adaxial surface uppermost on damp filter paper in 2 petri-dishes at room temperature. Drops of 4%(w/v) bacto-peptone containing 100 mM-sodium chloride were placed on one set of lobes, while the others were treated with 4%(w/v) bactopeptone only, as a control. Care was taken to prevent the fluid contacting the cut edges of the lobes.

After 2 h lobes were rinsed in distilled water (by flushing with a squeeze-bottle away from the cut edge) and treated as in a) and b) above, except at $4^{o}C$ and without vacuum.

From both treatments tissue was washed, dehydrated, and embedded in Spurr's resin. Sections were examined with and without post-staining with alkaline lead II citrate.

8) Thin-section Histochemistry

Serial sections were cut from material embedded in Epikote resin using a diamond knife and picked up on uncoated 200 mesh nickel grids. Material embedded in Epikote proved much better for this work than that set in Spurr's resin. Before enzyme-digestion treatment, sections were pretreated by immersing the grid in 3% (w/v) hydrogen peroxide solution at room temperature for 10 min, as proposed by Monneron and Bernhard (1966).

1. Lipase. A solution of 50,000 U ml^{-1} of lipase EC3.1.1.3 triacylglycerol acylhydrolase (SCC Type VII from Candida cylindracea) was prepared in 50 mM-sodium phosphate buffer (pH7.3). Grids were immersed in this and incubated for 20 - 240 min at 40°C. They were then washed in distilled water.

2. Protease. A solution of 3,500 U ml^{-1} of pronase (BDH from Streptomyces griseus) was prepared in distilled water brought to pH7.4 with sodium carbonate solution. Sections were incubated for 2 - 8 h at 40°C. They were then washed in distilled water.

Sections were post-stained with uranyl acetate and lead II citrate or viewed without further staining.

9) Scanning Electron Microscopy

Following fixation and post-fixation as in scheme (iii), material was dehydrated through a graded ethanol series and through an ethanol : acetone series (1:3, 1:1, 3:1) into two

changes of pure dry acetone. It was then dried in a Polaron E3000 critical point drying apparatus, flushing several times with liquid carbon dioxide (Air Products, Bracknell).

Pieces of tissue were mounted with double-sided Sellotape on aluminium specimen stubs and coated with about 20 nm of vaporized gold in a Polaron E5000 diode sputtering system, using an argon (Air Products, Bracknell) atmosphere. Stubs were dabbed with silver conducting paint to decrease charging and examined on a Cambridge Stereoscan IIa fitted with a dynamic focus unit, in the Clarendon Laboratory, Oxford, using 30 kV accelerating voltage and a 200 µm aperture. Some material was also examined on a Cambridge S150 at Cambridge Scientific Instruments Ltd., Cambridge. Images on the Stereoscan IIa were recorded on Ilford HP4 film, developed in Kodak 1D-11, fixed in Hypam (1 + 4) and printed as described previously. Material viewed on the S150 was recorded on Polaroid film.

10) High-voltage Electron Microscopy

This was used in an attempt to clarify (a) the ramifications of the labyrinthine cell walls and (b) the localization of cytochemical products.

Material was prepared as described previously. Semi-thick sections of 0.5, 1.0, and 1.5 µm were cut with glass knives on a Reichart OMU2 ultramicrotome using the manual fine advance. The sections were picked up on both coated and uncoated copper grids, stained for 30 - 45 min with alkaline lead II citrate, and viewed on an AEl EM7 electron microscope in the Department of Metallurgy, Oxford, at 1000 kV,

using a 100 μm aperture. Photographs were taken on cut sheets of Kodak electron microscope film 4489 and processed as above.

11) Light Microscopy

Hand-cut sections were prepared from fresh lobes held in pith and sliced with a razor-blade. Those of suitable thickness were selected and stained as follows.

1. Suberin (Johansen, 1940). Sections were cut into 70%(v/v) ethanol and then stained for 20 min in a saturated solution of Sudan IV in 70%(v/v) ethanol at room temperature. They were then treated for 3 - 5 min in 50%(w/v) chromic acid in the cold and macerated for 3 - 4 min in cold 10M-potassium hydroxide solution. This was warmed slowly to boiling, boiled a few minutes then cooled. Tissue was washed in distilled water and dried down onto glass slides.

Deposits of suberin are dark yellow from the reaction of phellic acid, which does not occur in cutin.

2. Protein (Johansen, 1940). Sections were cut into distilled water, stained 15 - 20 min with aqueous mercury II chloride-bromophenol blue solution (see 2:II:7) at room temperature and washed for 20 min in 0.5%(v/v) acetic acid. They were then immersed in distilled water for 3 - 5 min (approx. pH6.3) and dried down onto glass slides.

Protein stains blue.

3. True fats (Johansen, 1940). Cut sections into 70%(v/v) ethanol. Stain for 20 min in a saturated solution of Sudan IV in 70%(v/v) ethanol. Rinse in absolute ethanol and dry down

onto glass slides.

A bright red coloration shows true fats, although this fades to a red-brown on storage.

4. Tan (Johansen, 1940). Cut sections into water. Stain for 10 - 20 min in fresh, aqueous 10%(w/v) iron III chloride solution, containing a trace of sodium carbonate. Rinse in distilled water and dry down onto glass slides.

Tannins acquire a blue-green colour.

Slides were viewed on a Zeiss light microscope and recorded on Kodak Kodachrome II (artificial light sensitive) film.

12) Quantitative Image Analysis

To clarify the changes that occur in the gland-cells following stimulation, glands were stimulated with 4%(w/v) bactopeptone solution and lobes prepared for electron microscopy after 0,2,4 and 6 days. Whole cells were photographed at 2.5 - 6.3 k on the electron microscope and montage images constructed of final magnification 10.0 - 17.6 k.

1. Whole-cell quantitation. The grid-line method of Loud (1962) was used to analyse the cellular structures. A grid of narrow black lines spaced 20 mm apart was ruled on clear plastic sheet. This was overlaid on the photograph and held in place with Blu-tack (Bostik, Leicester) to prevent movement during analysis. Each photograph was analysed at 3 (or, initially, 5) orientations. The relative volume occupied by each organelle was obtained by expressing the length of line

overlying that organelle as a percentage of the total length. Endoplasmic reticulum was analysed by counting the number of crossings it makes with the grid lines and using Buffon's formula to calculate the total size of the population thus sampled.

2. Labyrinthine wall analysis. Black line-drawings were made of the perimeters described by the labyrinthine wall and the middle lamella of individual cells or parts of cells. The perimeter lengths and enclosed areas (with the whole cells) of these were measured using an IMANCO Quantimet 720 analytical system in the Department of Anatomy, Oxford. Measurements were made on whole cells and on each individual face of the cell, so as to examine the polarity of the transfer system. The ratio of the length of labyrinthine wall to the length of the middle lamella over a segment was used to assess the degree of elaboration present and called the amplification factor.

An electron micrograph, however, only renders a 2-dimensional image of the transfer wall areas. To extend this to 3 dimensions the total area of each type of surface contributing to the transfer-wall system is required. To obtain this, serial sections 2.0 μm thick were cut through whole glands of tissue embedded in Spurr's resin on a Reichart OMU2 ultramicrotome. Sections were cut on dry glass knives one at a time, picked up on a wet sable brush, and transferred to seried drops of water on a clean glass microscope-slide. By rapidly drying down at 80°C on an LKB2208 multiplate most sections dried completely flat and were firmly adhered to the slide.

Sections were viewed at x 400 magnification on a Zeiss light microscope fitted with a camera lucida. Pencil drawings were made of all the cell-walls and the lengths required for subsequent analysis blackened. Lines were measured using the Quantimet analyser. A 100 µm calibration line was used to convert the drawing lengths to µm of cell wall and hence to the area present in the 2.0 µm section. The area of each surface was obtained by summation across all relevant sections. The volume of each part of the gland was similarly obtained. Five glands were analysed.

III FEEDING AND COLLECTING METHODS

1) Stimulating the Glands

Mature healthy traps were initially fed 200 - 400 µl of a 2 - 4% (w/v) bactopeptone solution using an Eppendorf pipette and stimulated to close by agitation of the trigger hairs. Traps which failed to close rapidly were not used in the experiment.

On each day following stimulation any fluid held within the trap was withdrawn (see below) and replaced with 100 - 200 µl of the stimulating solution. Normally secretion would start within 24 h of stimulation, but in some cases repeated stimulation over 2 - 3 days was needed. This was probably due to the fluid evaporating in the highly desiccating conditions of the growth cabinet while the lobe was still in the 'closing' position (Ashida, 1934) and before stimulation could occur. Secretion never commenced before traps adopted the 'narrowing' position (Ashida, 1934) but it then continued for 5 - 11 days. In all experiments at least 8, and usually more, traps were used for each treatment.

2) Collecting the Secretion

Secretion was withdrawn from closed traps using a 20 µl glass disposable micro-pipette (Camlab, Cambridge: American Hospital Supply Corporation, Miami, USA) as described previously (Robins, 1975; Robins, 1976). The trap was carefully prised open with fine forceps and held slightly ajar. Fluid was drawn into the micro-pipette by gently sucking on

the end of a rubber tube fitted to the pipette-holder and blown out into a plastic micro-centrifuge tube. This was repeated until the trap was dry. Very little fluid was lost this way and it enabled an accurate measurement of the volume present to be made as it was collected. When the micro-pipette was not fully filled then the volume was obtained by measuring the length filled. Micro-pipettes of 50 µl capacity were tried but found to be too large and to damage the leaf tissue.

Secretion was stored frozen at -20^{o}C until required for analysis. It was collected so that the secretion from each of the days following stimulation was kept together, rather than that collected on each calendar day.

3) Estimation of the Area of the Traps

Because of the considerable variety in size and shape of the traps, no standard area measurement could be used. Each individual trap, therefore, in every set of measurements, had its area estimated in order to make the results directly comparable. Measurements were made of the external perimeters and the length across the arc at the top and bottom edges of closed traps while they were secreting.

The shape of each trap was reconstructed on graph paper which was cut into 800 mm^2 sheets, each of which bore the drawing of one trap. These sheets were weighed twice then the area of the lobe was excised. This was weighed 3 times and the mean mass was used to calculate the area of paper, based on the mass of the original 800 mm^2 sheet. This value

was doubled to obtain the area of the adaxial trap surface.

The error introduced by weighing was less than 0.5% and that from measuring and construction estimated at less than 5%.

4) Radiolabelling of the Secreted Protein

To examine the possibility that secreted protein was synthesized de novo prior to secretion and was not entirely from a stored source, experiments were conducted to investigate the incorporation of radioactivity into protein which was subsequently secreted during the same cycle.

Experiments were performed using mature leaves from healthy plants in both the growth cabinets and the greenhouse. Traps were fed 200 - 300 µl of stimulating solution containing radiolabelled L-leucine as detailed below.

1. To check for contamination by micro-organisms, traps were fed either:

(a) 4%(w/v) bactopeptone containing 5 µCi ml^{-1} L-[4,5-^{3}H]-leucine)

or

(b) 4%(w/v) bactopeptone containing 5 µCi ml^{-1} L-[4,5-^{3}H]-leucine, 0.05%(w/v) Benlate (50%(w/w) benomyl: Du Pont de Nemours & Co. Ltd., USA) fungicide and 1000 I.U. ml^{-1} benzylpenicillin.

Solution was administered daily and secretion withdrawn as described previously. Secretion was run separately on a Sephadex G-25 column (see 2:IV:1). The fractions collected were tested for radioactivity, protein and enzymatic activity

(see 2:V).

2. To examine the nature of the association of radiolabel with the peaks observed in 1. traps were fed with 4%(w/v) bactopeptone containing 2.5 μCi ml^{-1} L-[U-^{14}C]-leucine. Secretion was collected daily and further stimulating solution administered.

1 ml of secretion collected over several days was divided into 2 x 0.5 ml portions. One was treated with 100 μl 50%(w/v) trichloroacetic acid (TCA), the other with 100 μl buffer. Alternatively 4 volumes of absolute ethanol or buffer were added. After leaving for 20 min at room temperature, both samples were centrifuged for a few minutes on a Zentrifuge or 5 min on an MSE bench centrifuge at '8', and the supernatant withdrawn. This was run separately on a Sephadex G-25 column and 1 ml fractions collected (see 2:IV:1). These were assayed for radioactivity, acid phosphatase activity, and protein content (see 2:V).

5) Treatment with Inhibitors of Metabolic Processes

1. Disrupters of the secretory mechanism. To examine the effect of certain metabolic poisons known to act on secretory processes (Allison and Davies, 1974), the plants were stimulated as above but in the presence of the following compounds:

(a) Colchicine. A 4%(w/v) bactopeptone solution containing 1 mM- or 0.1 mM-colchicine (Koch-Light Laboratories Ltd., Colnbrook) was used.

(b) Cytochalasin B. A 4%(w/v) bactopeptone solution containing cytochalasin B at 5 μg ml^{-1} with 0.5%(v/v) dimethyl sulphoxide (DMSO) or 10 μg ml^{-1} with 1.0%(v/v) DMSO was used.

The secretion was collected and assayed for protein and enzymatic activity. Traps stimulated normally were used as a control.

2. Inhibition of protein synthesis. Healthy mature traps were stimulated to secrete in the presence of L-[U-^{14}C]-leucine and varying concentrations of cycloheximide. Stimulating solutions containing 1.125 μCi ml^{-1} L-[U-^{14}C]-leucine and 10^{-8},10^{-7},10^{-6}, and 10^{-5}M-cycloheximide were prepared. The control was identical but lacked cycloheximide, and an identical series of non-radioactive solutions was also made.

25 traps were used, 5 (randomly-selected) for each treatment. Initially these were fed 300 - 600 μl of the appropriate non-radioactive stimulating solution but after the first day on which secretion occurred they were fed 200 μl of the appropriate radiolabelled solution. Feeding was continued for 6 days and the secretion was collected and stored frozen at -20^{o}C. This was assayed for the amount of protein, radioactivity, and acid phosphatase and protease activities present. The amount of radiolabel in the protein content was also examined by precipitation with 10%(w/v) TCA solution, final concentration.

6) Density Labelling of the Secreted Protein

The technique of density labelling to demonstrate de

novo protein synthesis is often applied to systems where the synthesis of a particular enzyme, which may be induced by a stimulus at a known moment, is to be examined. By supplying a heavy isotope, either ^{2}H or ^{15}N, in a suitable form for incorporation into protein, the newly-synthesized enzyme may be detected by a shift in its buoyant density under isopyknic ultracentrifugation on a caesium chloride gradient.

^{15}N-label may be supplied as free amino acids, but these are extremely costly. ^{2}H-label, when supplied as $^{2}H_2O$, is rapidly incorporated into the amino acid pool by transamination reactions which assimilate the label at the C-1 position where it is not readily exchangeable. $^{2}H_2O$ was used for this work. Recently Lamb et al (in press) have demonstrated that the concentration of $^{2}H_2O$ used is crucial, as $^{2}H_2O$ itself is an inhibitor of protein synthesis and if exhibited at too high a concentration may adversely affect the process being studied.

1. Low $^{2}H_2O$ concentration. Mature healthy leaves of plants growing in the greenhouse were fed 200 - 400 µl of a 4%(w/v) bactopeptone solution in 50%(v/v) $^{2}H_2O$. Controls were treated simultaneously with 4%(w/v) bactopeptone in pure $^{1}H_2O$. Juice was collected for 4 days following stimulation and stored at -20°C.

2. High $^{2}H_2O$ concentration. Traps were treated as in 1. but with 4%(w/v) bactopeptone in 80%(v/v) $^{2}H_2O$. Secretion was collected separately for each of 8 days following stimulation and stored at -20°C.

The incorporation of label was investigated by isopyknic

ultracentrifugation (see 2:IV:4). Material collected after 80%(v/v) $^{2}H_{2}O$ feeding was assayed for protein content and enzymatic activity (see 2:V).

IV SEPARATION METHODS

1) Gel Fractionation

Molecular-sieve separation was used (a) to 'clean' crude secretion for enzymatic analysis and (b) to separate unreacted radiolabel from that which had been incorporated into secreted protein.

A column of Sephadex G-25 (176 x 10 mm) was thoroughly washed in running buffer, which was normally either 50 mM- or 100 mM-sodium acetate (pH5.2). Crude secretion (0.5 - 1.0 ml) was carefully layered onto the top, run into the column and overlayered with buffer. The column was fitted with a constant-head buffer reservoir and 28-drip fractions (approx. 1 ml) were collected.

The running-position of protein was calibrated using a 0.01%(w/v) solution of Blue Dextran 2000 in buffer and a 1 mg ml^{-1} Bovine Serum Albumin (BSA) solution in buffer (Fig. 2.1). The latter was assayed by the extinction of the fractions at 280 nm on a Beckman DB spectrophotometer. The position of the protein peak was checked on each run by measuring the extinction at 280 nm.

Fractions were either individually assayed for enzymatic activity or radioactivity (see 2:V) or the proteinaceous ones were pooled and used for other purposes.

2) Polyacrylamide Gel Electrophoresis (PAGE)

Disc PAGE was used as an analytical separative method

(a) to investigate the number of proteins present in the secretion, (b) to examine radiolabel incorporation into these proteins, and (c) to examine the enzymatic activity of some of these proteins.

1. Ordinary disc PAGE. Disc gels were prepared from a stock solution containing 29.2%(w/v) acrylamide (BDH: specially purified for electrophoresis) and 0.8%(w/v) bis (N,N'-Methylenebisacrylamide : Eastman Organic Chemicals, New York, USA) and polymerized with ammonium persulphate (APS) and Temed (N,N,N',N'-Tetramethylethylenediamine) with and without sodium lauryl sulphate (SDS) as follows:

Component	Stock concentration	Volume used (ml)	Final concentration
acrylamide	30%(w/v)	4.0	6.45%(w/v)
APS	0.5%(w/v)	1.1	0.03%(w/v)
Temed	0.5%(v/v)	1.1	0.03%(v/v)
SDS	5.0%(w/v)	0.4 (0.0)	0.11 (0.0)%(w/v)
buffer†	100 mM	12.0 (12.4)	65 (67) mM
		18.6	

†either 100 mM-sodium acetate (pH5.0)

or 100 mM-Tris/hydrochloric acid (pH8.9)

After mixing acrylamide and buffer, the solution was degassed for about 20 min under a water vacuum pump. The other components were added, mixed in and the liquid gel solution syringed into clean glass tubes (5 x 70 mm or 5 x 110 mm) using a disposable plastic pipette fitted with a Luer filling needle (No. N118). The tops were overlaid with water and the gels were left to polymerize overnight.

Electrophoresis was normally conducted in ice-cold buffer using 4 or 6 mA per tube. Disc tubes were pre-run in buffer for 2 - 3 h. 20 - 50 µl crude secretion was mixed with 5 or 10 µl 0.004%(w/v) bromophenol blue solution containing 40%(w/v) sucrose. This mixture was applied to the upper ends of disc tubes and overlaid with running buffer, which was the same as that used in setting the gels. The same buffer was used in each electrode chamber.

Electrophoresis was conducted for 40 - 100 min, depending on the current used and the total length of gel. The run was terminated just before the bromophenol blue marker reached the end of the gel. The gel was extruded by syringing distilled water down the sides and the length was measured before treatment by one of the following methods:

(i) Total protein stain. Gels were stained overnight in 0.1 or 0.5%(w/v) Coomassie brilliant blue R in a fixative solution of propan-2-ol/acetic acid/water (25:10:65), destained until clear in several changes of a solution of methanol/acetic acid/water (14:7:79), and the lengths and band positions of the gels recorded.

(ii) Acid phosphatase stain. Gels were run in buffer at pH5.0 and then either:

(a) incubated for 30 min in acid phosphatase substrate solution (see 2:V:3) at room temperature, plunged into 1 M-sodium hydroxide, watched as the yellow colour band appeared, and recorded.

or (b) sliced using a grooved metal block and a razor blade into segments 0.2 mm thick, incubated for 2 h in 0.5 ml of acid phosphatase substrate solution (see 2:V:3), stopped

by the addition of 0.5 ml 0.1 M-sodium hydroxide and the extinction at 400 nm read on a Unicam SP600 spectrophotometer.

(iii) Radioactivity. Initially it was attempted to measure this by the method of Gezalius (1977), using dehydration in absolute ethanol followed by toluene impregnation and scintillation counting with butyl-PBD scintillant (12 g l^{-1} toluene). The method, however, did not prove satisfactory with a 7%(w/v) gel (it was developed for a 2%(w/v) gel) but after correspondence, Dr Gezalius developed a method suitable for a 7%(w/v) gel (Gezalius, personal communication). By this time, however, the Lumac scintillant had been investigated and found satisfactory and radiolabel was counted in gels as described later (see 2:V:4).

2. Zymograms for acid protease activity. The method used was essentially that of Amagase (1972) but modified as outlined below.

Polyacrylamide gels were prepared as above, without SDS, but with 1.0%(w/v) casein in 100 mM-sodium acetate buffer (pH5.0) replacing the ordinary buffer. The gels were polymerized as before but not pre-run before use.

20 - 60 µl crude secretion mixed with 10 µl 0.004%(w/v) bromophenol blue solution containing 40%(w/v) sucrose was layered onto the tubes and overlayered with the anode chamber running buffer (50 mM-sodium acetate (pH5.0) containing 0.75%(w/v) casein). The cathode chamber buffer was 50 mM-sodium acetate (pH5.0) without casein. Electrophoresis was conducted for 40 min at 4 mA per tube.

Gels were removed from the glass support rods and

soaked for 30 min in 0.1 M-acetic acid at room temperature. They were then incubated for 60 min in a saturated atmosphere of water vapour at 40 - 45°C before being left overnight in 0.5%(w/v) Coomassie brilliant blue R staining solution (see above). After destaining, the gels were photographed, the light areas indicating proteolytic activity.

3) Polyacrylamide Gel Isoelectric Focussing

This technique was adopted after obtaining satisfactory separation of proteins by conventional electrophoretic procedures had proved difficult. Disc gels were prepared, as outlined below, from 30%(w/v) stock acrylamide (29.2%(w/v) acrylamide plus 0.8%(w/v) bis) and containing LKB Ampholines to establish the pH gradient over the range pH3.5 - 7.0. Components were mixed as follows:

Component	Stock concentration	Volume used (ml)	Final concentration
acrylamide	30%(w/v)	1.2	4.0%(w/v)
ampholine 1809-111 (pH3.5 - 5.0)	as supplied	0.6	-
ampholine 1809-121 (pH5.0 - 7.0)	as supplied	0.6	-
APS	10%(w/v)	0.03	.033%(w/v)
nonidet P40	10%(w/v)	0.4	0.45%(w/v)
water	pure	6.17	-
		9.00 ml	

All solutions, except APS and nonidet P40, were mixed and degassed by water-vacuum pump for about 10 min. After

adding these components, the gel solution was syringed into clean glass tubes (5 x 110 mm), overlayered with butan-2-ol and left at least 3 h to set. The butan-2-ol was removed and the top rinsed with water before use. The lower end of the gel tube was covered with muslin, held in place by a rubber grommet.

The volume of sample to be run (100 - 400 µl) was made to 20%(w/v) sucrose by the addition of solid sucrose and run onto the top of the gel. This was overlayered with 50 µl of 5%(w/v) sucrose solution and then overlayered with the top reservoir (cathode) running solution, 0.1 M-sodium hydroxide (pH12.0). The lower reservoir (anode) running solution was 0.01 M-orthophosphoric acid (pH1.98).

Isoelectric focussing was conducted at 50 - 150 V per tube (constant voltage) for 3 - 4 h followed by 1 - 2 h at twice the voltage per tube to sharpen the bands. Gels were extruded and subjected to one of the following treatments. For treatments 2 - 5 gels were held in an aluminium block with narrow slots 2.0 mm apart and sliced with a razor blade.

1. Protein bands. Unsliced gels were placed in staining solution and incubated at 60°C for 45 min. Destaining was performed in a solution of water/95%(v/v) ethanol/acetic acid (8:3:1).

Stain:

0.09 g Coomassie brilliant blue R was dissolved in 28 ml methanol then poured into 57 ml water and mixed rapidly. 2.8 g 5-sulphosalicylic acid (Fisons Scientific Apparatus, Loughborough) and 9.4 g TCA were added and mixed until dissolved. The solution was used while fresh.

2. pH gradient. The gels were sliced as above and 2 x 2.0 mm adjacent segments were placed in 2.0 ml distilled water, left for 3 - 4 h, and the pH read on a Pye Unicam 290 pH meter calibrated with BDH standard buffer solutions.

3. Acid protease. The gels were sliced as above and 1 x 2.0 ml slices incubated for 1 - 2 h in 0.5 ml of a 0.5%(w/v) casein solution in 50 mM-sodium acetate buffer (pH5.0) at 30°C with shaking. The reaction was stopped by adding 200 µl 50%(w/v) TCA and the tubes left to stand in the cold for 20 min. After centrifugation, 0.5 ml of the supernatant was withdrawn and the concentration of free amino acids assayed by the ninhydrin reaction (see 2:IV:2).

4. Acid phosphatase. The gels were sliced as above and incubated for 1 - 2 h in 0.5 ml of acid phosphatase substrate solution (see 2:V:3) at 30°C without shaking. 300 µl was withdrawn into 1.0 ml 0.1 M-sodium hydroxide and the extinction at 400 nm read on a Unicam SP600 spectrophotometer.

5. Radioactivity. Each 2.0 mm segment of the sliced gel was prepared for liquid scintillation counting (see 2:V:4).

4) Isopyknic Ultracentrifugation

Isopyknic ultracentrifugation was performed on a caesium chloride gradient, in buffer, in 10 ml capacity polypropylene MSE centrifuge tubes. The gradient was established by centrifugation on an MSE Superspeed 50 ultracentrifuge using a fixed-angle rotor at 42,000 rev min^{-1} (110,000 g) for 32 - 46 h at 5.5°C.

1. Preparation of material. 0.8 - 1.0 ml of secretion was thawed and cleaned on a Sephadex G-25 column at room temperature in running buffer (see below). The six fractions (6 ml) containing the protein peak were collected together and to this was added 60 µl β-galactosidase to act as an internal marker (Acton and Schopfer, 1975). The preparations were kept on ice until required. A control and a treated sample were always prepared together.

2. Preparation of the gradient. Tubes were pre-cooled in ice. Into each tube was pipetted either (a) 3.6 ml of an 82%(w/v) caesium chloride solution (BDH : for ultracentrifuge work) overlaid with 3.6 ml of a 20%(w/v) solution or (b) 3.6 ml of an 82.5%(w/v) solution overlaid with 3.6 ml of a 22%(w/v) solution. Sample was applied over this with a syringe, the caps locked onto the tubes and further sample syringed in until the tubes were full. The balance of tubes was checked and, if necessary, corrected to a tolerance of ±100 mg. They were then centrifuged as above.

Gradients were initially prepared using system (a) but these gave a skew acid phosphatase peak. System (b) was introduced to correct for this.

3. Choice of pH. Initially material was prepared in 100 mM-sodium acetate buffer (pH5.0) as used elsewhere. It was soon apparent, however, that at this pH the interval marker, β-galactosidase, lost virtually all its activity. This was confirmed by testing the residual activity of this enzyme at pH5.0 and pH7.0 in the presence of 0, 20, 40, and 60%(w/v) caesium chloride after storage at $4^{o}C$ for 24 h and 48 h (Fig. 2.2). The activities of acid protease and acid

phosphatase from the secretion were also tested under the same conditions (Fig. 2.2). The buffers used were 100 mM-sodium acetate (pH5.0) and 100 mM-sodium dihydrogen phosphate (pH7.0), and the enzymes were assayed by the standard procedures (see 2:V).

From this experiment it was decided that 100 mM-sodium dihydrogen phosphate buffer (pH7.0) should be suitable, but a gradient run with this buffer proved unsatisfactory. Gradients were therefore tried using the caesium chloride solutions in (b) above and at pH6.0 with 100 mM-acetic acid/ Tris buffer and at pH8.1 with 100 mM-Tris/acetic acid buffer. Both of these proved satisfactory but a higher acid phosphatase activity was retained at pH6.0 with ample β-galactosidase and so this buffer was used in future.

4. Fractionation of gradient. At the termination of a centrifugation, tubes were removed from the rotor and placed vertically in ice. Fractionation was performed immediately. The tube to be fractionated was held in a clamp 30 - 40 cm above the bench and a long hypodermic needle, attached to a nylon syphon tube primed with buffer, was placed down the centre of the centrifuge tube. The clamp on the syphon tube was released and 82 - 86 x 4 drop fractions collected in plastic micro-centrifuge tubes.

Every tenth fraction (10,20,30, etc.) was sealed and stored in the cold (4^{o}C) to be used to establish the shape of the gradient (see below). 20 µl aliquots were withdrawn from every third remaining fraction (1,4,7,11, etc.) and assayed for β-galactosidase activity (see 2:V:3) while the remainder of those fractions was assayed for acid phosphatase activity

(see 2:V:3). The remaining fractions were used either to assay for other enzymatic activities or to check the positions of the β-galactosidase and acid phosphatase peaks.

5. Shape of the gradient. The fractions used for this were allowed to warm up to the temperature of the room in which the measurements were made. The shape of the gradient was then established by measuring their refractive indices on an Abbé refractometer, illuminated by indirect sunlight and cleaned with diethyl ether.

5) Ion Exchange

Both cationic and anionic exchange resins were employed to examine whether the radiolabel associated with the proteinaceous fractions from gel filtration could be separated from the protein.

Two small columns were made in Pasteur pipettes, one containing Amberlite 1R-120 cation exchange resin in 50 mM-sodium acetate buffer (pH5.0) and the other containing Amberlite 1RA-400 anion exchange resin in 50 mM-Tris/acetic acid buffer (pH7.8). These were left for about 1 h during which they were washed with several changes of buffer. 1 ml of Sephadex G-25 eluant, collected from plants fed L-[U-^{14}C]-leucine in 4%(w/v) bactopeptone, was run into each column and washed through several times before being left for 1 h to equilibrate at room temperature. Columns were then drained and washed with 3 x 1 ml of buffer. A further 1 ml of buffer was placed in the columns which were left at 4^{o}C overnight.

The eluant and washings were pooled, brought to pH5.0 with 1 M-acetic acid and made up to 6 ml with 50 mM-sodium acetate buffer (pH5.0).

To each column was added 1 ml of the appropriate counter-ion buffer: 250 mM-Tris/acetic acid (pH8.0) to the 1R-120 and 250 mM-sodium acetate (pH5.0) to the 1RA-400. This was washed through several times as before and left 3.5 h to equilibrate at 4°C. Columns were then drained dry and washed with 3 x 1 ml distilled water. The eluant and washings were combined, brought to pH5.0 with 1 M-acetic acid or 1 M-sodium hydroxide and made up to 6 ml with distilled water.

Aliquots of each sample were analysed for protein content, protease activity, acid phosphatase activity and radioactivity (see 2:V).

6) Dialysis

Dialysis was also used to examine whether the radio-label in the proteinaceous peak from gel filtration could be separated from the protein and enzymatic activities.

Tubing was prepared by boiling in 1%(w/v) sodium hydroxide containing 0.1%(w/v) EDTA (ethylenediaminetetra-acetate) for 10 min and then washing in copious amounts of distilled water. Into a short length of this tubing was placed 2.5 ml of Sephadex G-25 eluant, collected from plants fed L-[U-^{14}C]-leucine in 4%(w/v) bactopeptone, and the tubing sealed with a double knot at each end. The solution was dialysed for 24 h against 300 ml of 100 mM-sodium

acetate buffer (pH5.0) at 4°C followed by a further 24 h against a fresh 300 ml of buffer.

A small amount of solid sodium hydroxide was added to the diffusate which was then boiled down to about 10 ml. Aliquots of the dialysis residue, which showed no change in volume, were assayed for protein content, protease and acid phosphatase activities and radioactivity (see 2:V). The whole volume remaining from the diffusate was assayed for radioactivity (see 2:V:4).

V ASSAY METHODS

1) Protein

Protein was measured by the method of Lowry et al (1951). 10 - 20 µl crude secretion was taken and made up to 500 µl with either distilled water or 50 mM-sodium acetate buffer (pH5.0). To this was added 2.0 ml fresh Lowry 'C' reagent, mixed, and left at room temperature. After 10 min, 200 µl 50%(v/v) Folin and Ciocalteu's phenol reagent (BDH : ordinary grade) was added, mixed and left 30 min at room temperature before reading the extinction at 750 nm on a Beckman DB spectrophotometer with distilled water or buffer respectively in the reference cell. A reagent blank and a standard (Fig. 2.3) over the range 0 - 100 µg BSA Fraction V (crystalline) were always included.

When the protein content of more dilute solutions was determined (eg. gel filtration fractions) 100 - 200 µl was used, made up to 500 µl with buffer and treated as above.

Reagents:

Lowry solution A: 2%(w/v) sodium carbonate in 0.1 M-sodium hydroxide

Lowry solution B: 1 part 5%(w/v) hydrated copper II sulphate
1 part 10%(w/v) hydrated sodium potassium tartrate
8 parts distilled water (mixed just before use)

Lowry solution C: 1 part B mixed with 50 parts A just before use.

BSA stock: 1 mg ml^{-1} in water stored frozen at $-20^{o}C$.

Previous workers (Scala et al, 1969) reported a high

background extinction at 280 nm in the secretion which they considered due to the presence of phenolics. Precipitation of the protein with 7%(w/v) TCA followed by dissolution of the precipitate in solution A made negligible difference to the final values obtained. It was therefore considered unnecessary to perform this step as a part of the routine assay.

2) Proteolytic Activity

1. Non-specific acid protease. General proteolytic activity was measured at pH5.0 using light white soluble casein as substrate and assaying for the ninhydrin-positive products.

a) Crude secretion. 1.0 ml of a 1%(w/v) casein solution was mixed with 1.0 ml of 100 mM-sodium acetate buffer (pH5.0) and pre-warmed to 30°C in a shaking water bath. 10 - 20 µl secretion was added and incubated for 60 min, after which the reaction was stopped by the addition of 1.0 ml 15%(w/v) TCA. This was left for 20 min either at room temperature or in ice and then centrifuged on an MSE bench centrifuge, speed 7 - 9, for 5 min.

Duplicate 1.0 ml samples of the supernatant were withdrawn and to each were added 0.5 ml 0.5 M-sodium citrate buffer (pH5.0) and 1.2 ml potassium cyanide-ninhydrin reagent, prepared at least 4 h before use (Yemm and Cocking, 1955). Tubes were thoroughly mixed, incubated for 20 min in a boiling water bath and cooled to room temperature. 3.0 - 5.0 ml 60%(v/v) ethanol were added and the extinction at 570 nm read on either a Beckman DB or Unicam SP600 spectrophotometer.

Secretion incubated without casein and casein incubated without secretion were used as controls. Boiling secretion to destroy enzymatic activity tended to greatly enhance the ninhydrin-reactivity, possibly due to autolysis as the secretion warmed up. A glycine standard was always included with a range of 0 - 10 µg glycine (Fig. 2.4).

In some later experiments it was found convenient to halve the amounts used and to incubate in micro-centrifuge tubes. In these cases, reaction was stopped by adding 200 µl 50%(w/v) TCA and subsequently spinning for 2 min on a Zentrifuge. Treatment was otherwise identical but half the amount of reagents was used.

Reagents:

potassium cyanide: 5.0 ml 0.01 M-potassium cyanide diluted to 250 ml with ethylene glycol monomethyl ether

ninhydrin: 5%(w/v) solution in ethylene glycol monomethyl ether

b) Assay of partially purified secretion. When partially purified secretion was assayed for protease activity 200 - 500 µl were used and the volume of buffer added was decreased accordingly. Purification was normally in 50 mM-sodium acetate buffer (pH5.0).

c) Inhibition studies. To try to examine the types of proteases present, the proteolytic activity of secretion was assayed in the presence of various inhibitors of proteolytic activity (Boyer, 1971).

1.0 ml 'clean' secretion was pre-incubated with 1.0 ml of inhibitor solution (see below) at 30°C for 1 h with shaking. To this was added 2.0 ml of 1% (w/v) casein in 100 mM-sodium acetate buffer (pH5.0) and incubated for 1 h. 1.0 ml was withdrawn into 1.0 ml 15% (w/v) TCA and left 25 min before centrifuging. The ninhydrin-positive product in the supernatant was then assayed as above.

Apart from the usual controls, others were included to allow for the effect of the various solvents on enzymatic activity and blanks were included to allow for the effect of the inhibitors on the ninhydrin reaction. Glycine was used as a standard. Papain and pepsin solutions were treated concurrently to verify the effectiveness of the inhibitors. Casein was also used as the substrate with these, but as a 1% (w/v) solution in the appropriate buffer (see below).

Reagents:

Reagent	Stock concentration	Final concentration	Solvent
Pepstatin	0.04 mM	0.01 mM	Dissolved in absolute ethanol then diluted to 10% (v/v) ethanol (Umezawa et al, 1970)
EDTA	80.0 mM	20.0 mM	Aqueous
PMSF	40.0 mM	10.0 mM	Dissolved in pure propan-2-ol then diluted to 20% (v/v) propan-2-ol (Fahrney and Gold, 1963)

Reagent	Stock concentration	Final concentration	Solvent
Iodoacetamide	2.0 mM	0.5 mM	Aqueous
Acetic anhydride	80.0 mM	20.0 mM	Aqueous
Trypsin Inhibitor	1 mg ml^{-1}	250 µg ml^{-1}	Aqueous
Pepsin (BDH : ordinary)	100 µg ml^{-1}	25 µg ml^{-1}	100 mM-hydrochloric acid (pH1.4)
Papain (BDH : ordinary)	100 µg ml^{-1}	25 µg ml^{-1}	100 mM-potassium dihydrogen phosphate/sodium hydroxide (pH6.8) containing 5 mM-cysteine

1 katal of activity is defined as the release of 1 mole equivalent of glycine s^{-1}.

2. Papain-type activity. The hydrolysis of α-N-Benzoyl-L-arginine methyl ester (BAME) was followed by examining the rate of change of extinction at 280 nm (Schwert and Takenaka, 1955). 100 µl substrate and 100 µl enzyme were added to 1.8 ml buffer and rapidly mixed. The change in extinction at 280 nm was followed for a few minutes on a Unicam SP600 spectrophotometer, fitted with an expanded scale, and then either (i) 100 µl EDTA reagent was added and the extinction at 280 nm followed as before

or (ii) 100 µl iodoacetamide reagent was added and the extinction at 280 nm followed as before.

The metal-chelator EDTA acts to stimulate papain by removing heavy metals which block the thiol site. Iodoacetamide is well known as an inhibitor of proteases with a free thiol group at the active site.

Aliquots of 50, 100 and 200 µl of a standard 200 µg ml^{-1} papain (BDH : ordinary) solution were used. This was tested with and without EDTA and iodoacetamide and also at the optimal pH for papain (6.75) and at the optimal pH for secretion (5.2). The assay was performed using crude secretion.

Reagents:

1 mM-BAME in 50% (v/v) methanol

50 mM-sodium acetate buffer (pH5.2) with and without 100 mM-calcium chloride

50 mM-disodium hydrogen phosphate buffer (pH6.75) with and without 100 mM-calcium chloride

0.5 mM-iodoacetamide in water

100 mM-EDTA (disodium salt) in water

1 mg ml^{-1} papain (BDH) in water without cysteine.

3. Chymotrypsin-type activity. The release of p-nitrophenol from N-CBZ-L-phenylalanine-p-nitrophenyl ester was followed spectrophotometrically at 400 nm. 1.15 ml of buffer was mixed with 250 µl substrate solution and warmed to 30°C. 100 µl crude secretion was added and incubated for 1 h at 30°C. 1.0 ml 0.1 M-sodium hydroxide solution was added and the extinction at 400 nm measured on a Unicam SP600 spectrophotometer versus buffer. A control was incubated with 100 µl buffer instead of secretion.

Substrate reagent:

2 mM-N-CBZ-L-phenylalanine-p-nitrophenyl ester in 1,4-dioxane

50 mM-sodium acetate buffer (pH5.2) with 100 mM-calcium chloride.

4. Trypsin-type activity. The method used involved the spectrophotometric detection of the hydrolysis of N-α-Toluene-4-sulphonyl-L-arginine methyl ester (TAME) (Hummel, 1959).

2.0 ml substrate solution was warmed to 30°C and 100 µl enzyme was added. The change in extinction at 280 nm was followed on a Unicam SP600 spectrophotometer fitted with an expanded scale. A control was used with buffer instead of crude secretion. Activity was tested at both pH5.2 and pH8.1.

Reagents:

1 mM-N-α-Toluene-4-sulphonyl-L-arginine methyl ester in buffer

50 mM-Tris/hydrochloric acid buffer (pH8.1) with 100 mM-calcium chloride

50 mM-sodium acetate buffer (pH5.2) with 100 mM-calcium chloride.

5. Leucine aminopeptidase. N-terminal exopeptidase activity was assayed by testing the activity of secretion versus L-Leucyl-β-naphthylamide, coupling the reaction to fast garnet GBC salt and following the reaction spectrophotometrically at 525 nm (Kolehmainen and Mikola, 1971).

4.9 ml buffer containing 1.4 ml substrate was warmed to 30°C then 700 µl crude secretion added. 1.0 ml samples were withdrawn at 0, 2, 5, 10, 15 and 25 min following the addition of enzyme. Each sample was added to 500 µl fast garnet GBC reagent. After 15 min the extinction at 525 nm was read on a Unicam SP600 spectrophotometer versus an incubated control.

Reagents:

2 mM-L-Leucyl-β-naphthylamide in 10 mM-hydrochloric acid

0.1% (w/v) Fast garnet GBC salt in 100 mM-sodium acetate buffer, (pH4.2) containing 10% (v/v) Tween 20.

25 mM-sodium acetate buffer (pH5.2).

6. Carboxypeptidase-type activity. The C-terminal exopeptidase activity of the crude secretion was assessed using substrates specific to both A-type and B-type carboxypeptidase activity.

a) A-type. Carboxypeptidase-A-type activity was examined spectrophotometrically by following the release of p-nitrophenol from N-CBZ-L-alanine-p-Nitrophenyl ester, a modification of the method of Visuri et al (1969).

1.15 ml buffer containing 200 µl substrate reagent was warmed to 30°C then 100 µl crude secretion was added and the mixture incubated for 1 h at 30°C. The reaction was stopped by adding 3.0 ml 0.1 M-sodium hydroxide and diluted as required with further 0.1 M-sodium hydroxide before the extinction at 400 nm was read against buffer on a Unicam SP600 spectrophotometer.

Reagents:

2 mM-N-CBZ-L-alanine-p-nitrophenyl ester in 1,4-dioxane

50 mM-sodium acetate buffer (pH5.2).

b) B-type. Carboxypeptidase-B-type activity was examined spectrophotometrically by following the hydrolysis of N-Benzoylglycyl-L-arginine (Wolff et al, 1962).

100 µl crude secretion was added to 200 µl substrate solution in 1.0 ml buffer and mixed well. The extinction at

223 nm was followed, against buffer, on a Unicam SP600 spectrophotometer fitted with an expanded scale.

Reagents:

10 mM-N-Benzoylglycyl-L-arginine in buffer

50 mM-Tris/hydrochloric acid buffer (pH8.1)

50 mM-sodium acetate buffer (pH5.2)

3) Other Enzymes

1. Acid phosphatase. The hydrolysis of p-Nitrophenyl disodium orthophosphate at pH5.0 was used to assay for acid phosphatase activity (Dingle, 1972). To 1.0 ml 5.5 mM-p-Nitrophenyl phosphate solution was added 1.0 ml 100 mM-sodium acetate buffer (pH5.0) and 50 µl 62.5 mM-magnesium chloride. This was prewarmed to 30°C and then 10 µl crude secretion was added. After 1 h incubation, the reaction was stopped by the addition of 1.0 ml 0.1 M-sodium hydroxide and the extinction read at 400/410 nm on a Beckman DB spectrophotometer or a Unicam SP600. If the colour intensity was too great then suitable dilutions were made using further 0.1 M-sodium hydroxide.

Substrate solution incubated without enzyme was used as a control and a standard curve over 0 - 200 nmoles p-Nitrophenol was used to obtain the enzymatic activity (Fig. 2.5). When more dilute solutions of secreted proteins were used then the amount of buffer added was decreased accordingly.

2. Peroxidase. The method of Devlin (Chance and Maehly, 1955) was used. The rate of utilization of hydrogen peroxide was followed by coupling the reaction to the conversion of

the colourless dye guaiacol to tetraguaiacol, a red dye with an optimal absorption at 470 nm.

2.0 ml 20 mM-guaiacol solution, 1.0 ml buffer and 10 µl 10 mM-hydrogen peroxide solution were mixed together. Enzyme solution, made up to 200 µl with buffer, was added and rapidly mixed. The extinction at 470 nm was followed for about 5 min. on a Unicam SP600 spectrophotometer, fitted with an expanded scale.

Crude secretion was assayed directly, using 50, 100, 200 and 400 µl secretion. A 1 µg ml^{-1} solution of Horseradish Peroxidase (SCC: Type II) was used as a standard (Fig. 2.6). The activity of the peroxidase was calculated using the molar extinction coefficient (ε) of tetraguaiacol = 26.6 cm^{-1} mM^{-1} and the fact that four moles of hydrogen peroxide are used per mole of tetraguaiacol made.

Reagents:

20 mM-guaiacol solution (0.22 ml in 100 ml water)

10 mM-hydrogen peroxide solution (0.113 ml 30% (w/v) hydrogen peroxide in 100 ml water)

100 mM-dipotassium hydrogen phosphate buffer (pH7.0)

100 mM-sodium acetate buffer (pH5.0).

3. Chitinase. Activity against colloidal chitin was assayed at pH5.0. A solution of colloidal chitin (poly-N-acetyl-D-glucosamine) was prepared by a modification of the method of Amagase et al (1972).

5.0 g of powdered crab-shell chitin was mixed with 150 ml cold concentrated hydrochloric acid and left for 3 days in the cold, with constant stirring. Insoluble matter

was removed by vacuum filtration through a coarse sinter funnel, the filtrate was diluted to 1000 ml with cold distilled water and left to stand overnight in the cold. During this period, a white flocculent precipitate settled to the bottom of the vessel. The clear supernatant was removed by a water suction-pump and the precipitate washed with distilled water. This was repeated twice daily for twelve washes, until the acidity decreased to about pH4.0. It was then made up to 300 ml with 100 mM-sodium acetate buffer (pH5.0) and contained a final concentration of chitin of 150.3 $\pm$ 2.7 mg ml^{-1}. The pH was adjusted, as required, before use with sodium hydroxide.

Vials were set up in a vigorously shaking water bath at 30°C as follows:

Vial	Colloidal chitin ml		SCC chitinase solution ml	Secretion ml	Buffer ml	
	pH5.0	pH6.0			pH5.0	pH6.0
1	4.0	-	-	0.5	0.5	-
2	-	4.0	0.5	-	-	0.5
3	4.0	-	0.5	-	0.5	-
4	4.0	-	-	-	1.0	-
5	-	4.0	-	-	-	1.0

Aliquots of 1.0 ml were withdrawn after 0, 4, 13 and 23 h and assayed for the production of N-acetyl sugars by the method of Aminoff et al (1952).

100 µl 0.5 M-sodium carbonate solution was added to each aliquot, left for 10 min. at room temperature then centrifuged on an MSE bench centrifuge at speed 9 for 5 min. Two by 400 µl aliquots were taken, 100 µl 0.5 M-sodium

carbonate plus 600 µl distilled water added to each and they were heated in a boiling water bath for exactly 4 min. and then cooled rapidly in ice. 1.0 ml glacial acetic acid and 1.0 ml 4% (w/v) 4-Dimethylaminobenzaldehyde (DMAB) in glacial acetic acid were added then left for 2 h at room temperature in the dark before the extinction at 590 nm was read against glacial acetic acid on a Beckman DB spectrophotometer.

A solution of 1 mg ml^{-1} N-acetyl-D-glucosamine (NAG) was used as a standard over the range 0-300 µg (Fig. 2.7).

4. N-acetyl β-D-glucosaminidase. This enzyme, like acid phosphatase, is considered to be a marker for lysosomes or lysosomal products (Sogawa and Takahashi, 1977). Activity was therefore examined against the substrate p-Nitrophenyl-N-acetyl-β-D-glucosaminidine, by spectrophotometrically following the release of p-Nitrophenol (Mega et al, 1970).

100 µl crude secretion was added to 200 µl substrate solution, pre-warmed to 30°C, then incubated at 30°C for 30 min. The reaction was stopped by adding 1.0 ml 0.01 M-sodium hydroxide and the extinction at 400 nm read on a Unicam SP600 spectrophotometer.

Reagents:

0.2% (w/v) p-Nitrophenyl-N-acetyl β-D-glucosaminidase in buffer

100 mM-sodium acetate buffer (pH4.5).

5. β-galactosidase. This enzyme was used as an internal marker in the isopyknic ultracentrifugation experiments (Acton and Schopfer, 1975).

20 µl of solution containing the enzyme was added to 1.0 ml of o-Nitrophenyl β-galactopyranoside reagent and left for 15 - 30 min at room temperature. 0.8 ml was withdrawn into 2.0 ml 10% (w/v) sodium carbonate solution to stop the reaction. The extinction at 410 nm was read on a Unicam SP600 spectrophotometer.

Reagents:

0.05% (w/v) o-Nitrophenyl β-galactopyranoside in 100 mM-sodium dihydrogen phosphate buffer (pH7.0).

4) Radioactivity

1. Aqueous samples. Both ^{14}C and ^{3}H were measured using the emulsion counting system of Turner (see Peng (1977) p.60) which forms a stable colloid with a 33% (v/v) aqueous content. Scintillant was prepared by mixing a solution of 0.2% (w/v) PPO (Koch-Light Laboratories, Colnbrook) plus 0.05% (w/v) POPOP (Koch-Light Laboratories, Colnbrook) in toluene with Triton X100 (BDH : scintillation grade) in the ratio 2:1.

The sample (usually 1.0 ml) was added to twice its own volume of scintillant and thoroughly mixed. Counting was normally conducted in polythene inserts in nylon scintillation vials on an ICN Tracerlab with double-channel counting. Each vial was counted 2 or 3 times.

In some cases the commercial scintillant 'Lumagel' was used, which allows a 17 - 25% (v/v) aqueous content. This gave an enhanced counting efficiency and was used when the total counts was expected to be rather low.

2. Gel samples. 2.0 mm slices of polyacrylamide gels, of wet weight about 100 mg, were soaked overnight at room temperature in 2.0 ml of a Lipoluma/Lumasolve scintillant mixture in polythene inserts. After gently mixing, these were counted for radioactivity as above.

Reagent: 10 volumes Lipoluma
1 volume Lumasolve
0.2 volumes water.

Figure 2.1 Sephadex G-25 ellution profiles

Samples, as detailed below, were run in 50 mM-sodium acetate buffer (pH5.0) and 28-drip fractions collected. All operations at room temperature.



1.0 ml of 1 mg ml^{-1} Bovine Serum Albumin (BSA) solution in running buffer;



1.0 ml of 5.0 µCi ml^{-1} L-[4,5-^{3}H]-leucine in 4%(w/v) bactopeptone;



marks the ellution range of Blue Dextran-2000

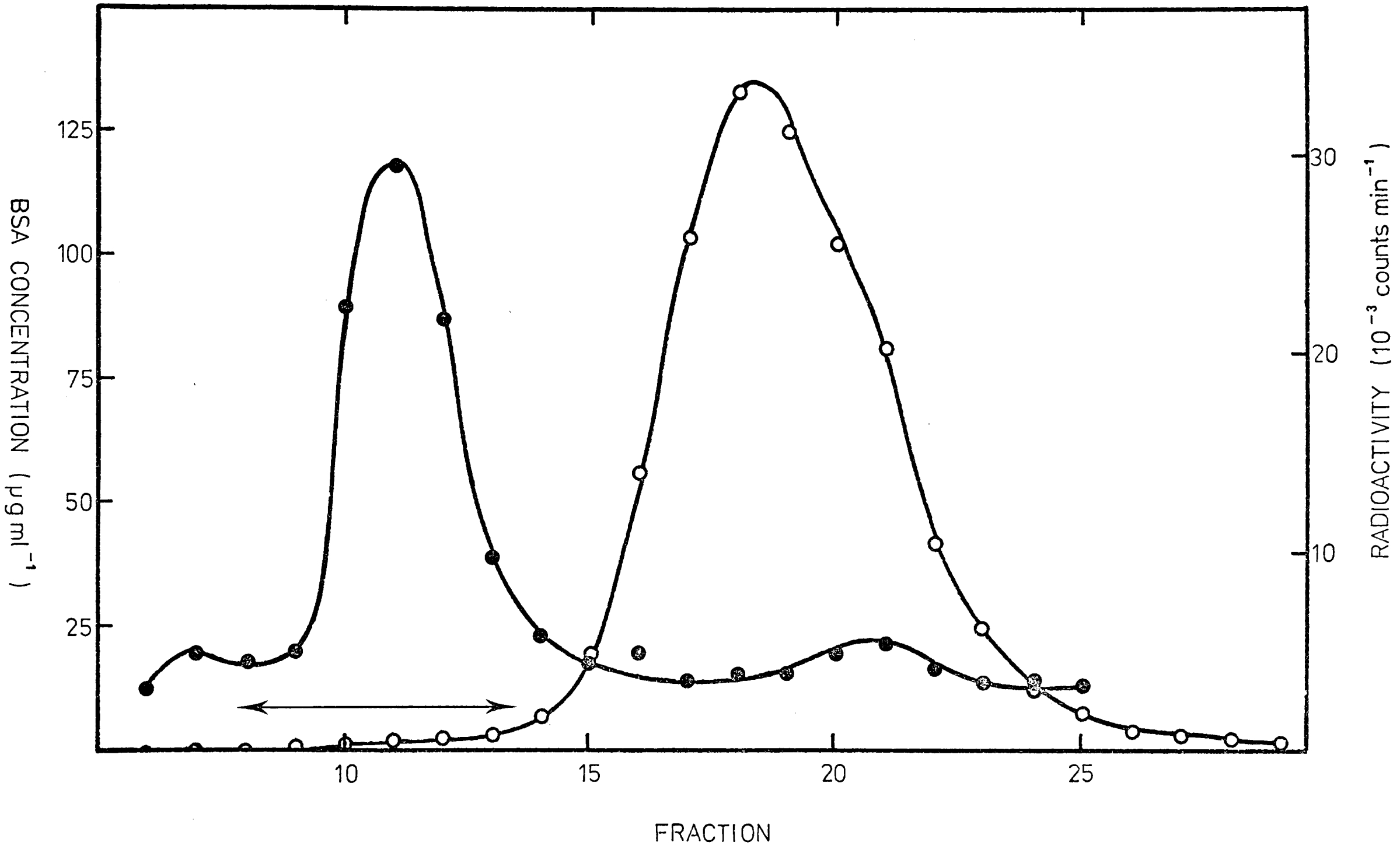

BSA CONCENTRATION (μg ml⁻¹)
125
100
75
50
25
RADIOACTIVITY (10⁻³ counts min⁻¹)
30
20
10
10
15
20
25
FRACTION

Figure 2.2 Stability of Acid Phosphatase, β-galactosidase and Acid Protease at different values of pH in the presence of Caesium Chloride

The enzyme solution contained 120 µl of β-galactosidase (BDH) per 1.0 ml secretion. 20 µl of this was added to caesium chloride solution in buffer, to a final volume of 300 µl. Each line represents the mean of preparations containing 0, 20, 40, and 60%(w/v) caesium chloride.

□ pH5.0
● pH6.0
■ pH7.0
○ pH8.1

Enzyme activities given as % of activity at zero time. These are:

		pH	Enzyme Activity [nkat.(ml enzyme solution)$^{-1}$]
a)	Acid Phosphatase	6.0	11.0
		8.1	9.6
b)	β-galactosidase	5.0	0.4
		6.0	12.2
		7.0	5.8
		8.1	1.2
c)	Acid Protease	5.0	2.8
		6.0	28.7
		7.0	4.4
		8.1	33.4

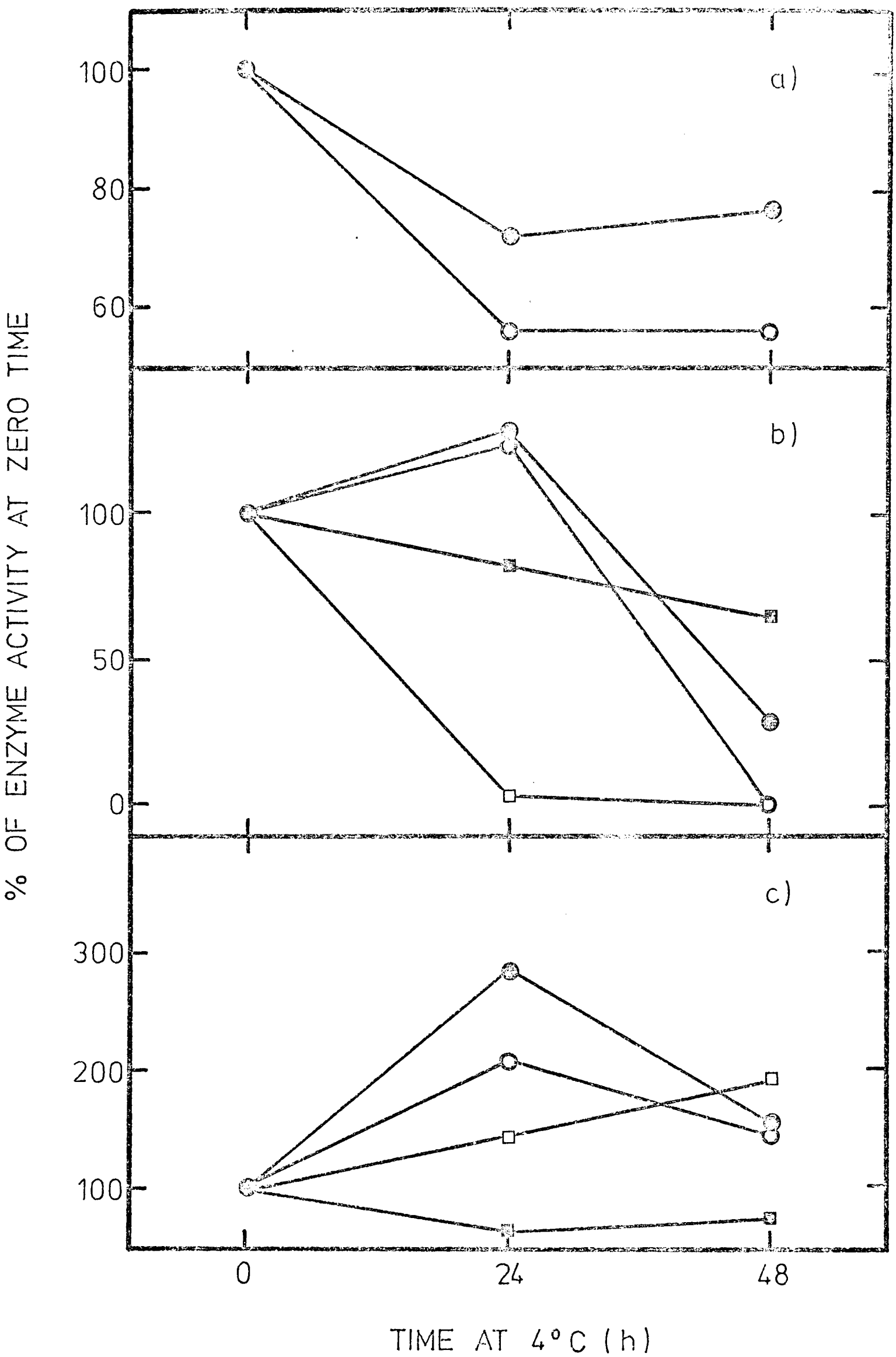
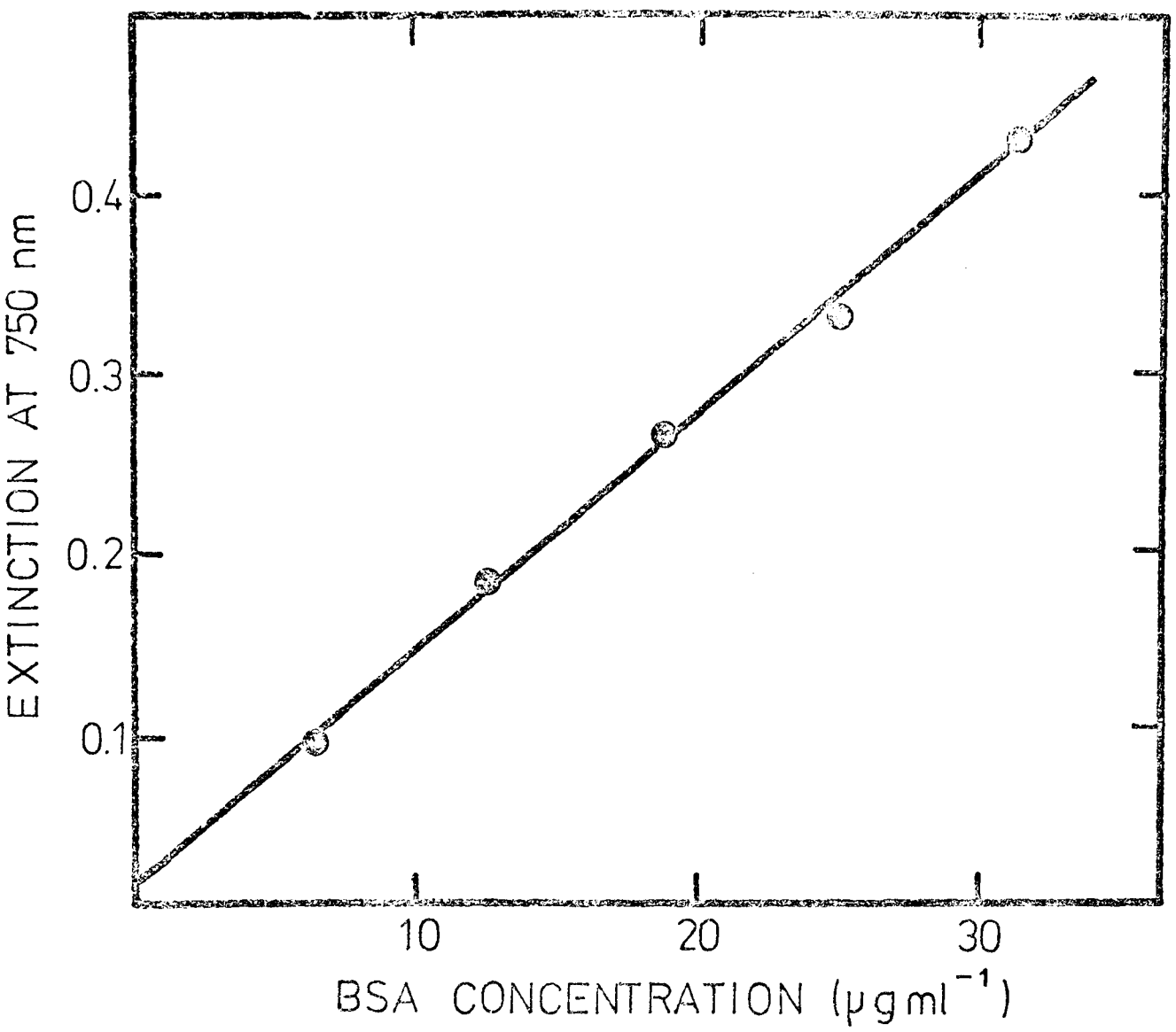

a)
b)
c)
100
80
60
100
50
0
300
200
100
0
24
48
% OF ENZYME ACTIVITY AT ZERO TIME
TIME AT 4°C (h)

Figure 2.3 Estimation of Protein

An example of a standard used for the estimation of protein by the method of Lowry et al (1951), as described in the text. Line drawn by linear regression. BSA present given as $\mu g\ ml^{-1}$ in final volume (here = 3.2 ml).

Figure 2.4 Estimation of Protease activity

An example of a standard used for the estimation of protease activity, using glycine and assayed using the method of Yemm and Cocking (1955) as described in the text. Line drawn by linear regression. Glycine present given as $nmol\ ml^{-1}$ in final volume (here = 4.25 ml).


EXTINCTION AT 750 nm
0.4
0.3
0.2
0.1
10
20
30
BSA CONCENTRATION (μg ml⁻¹)

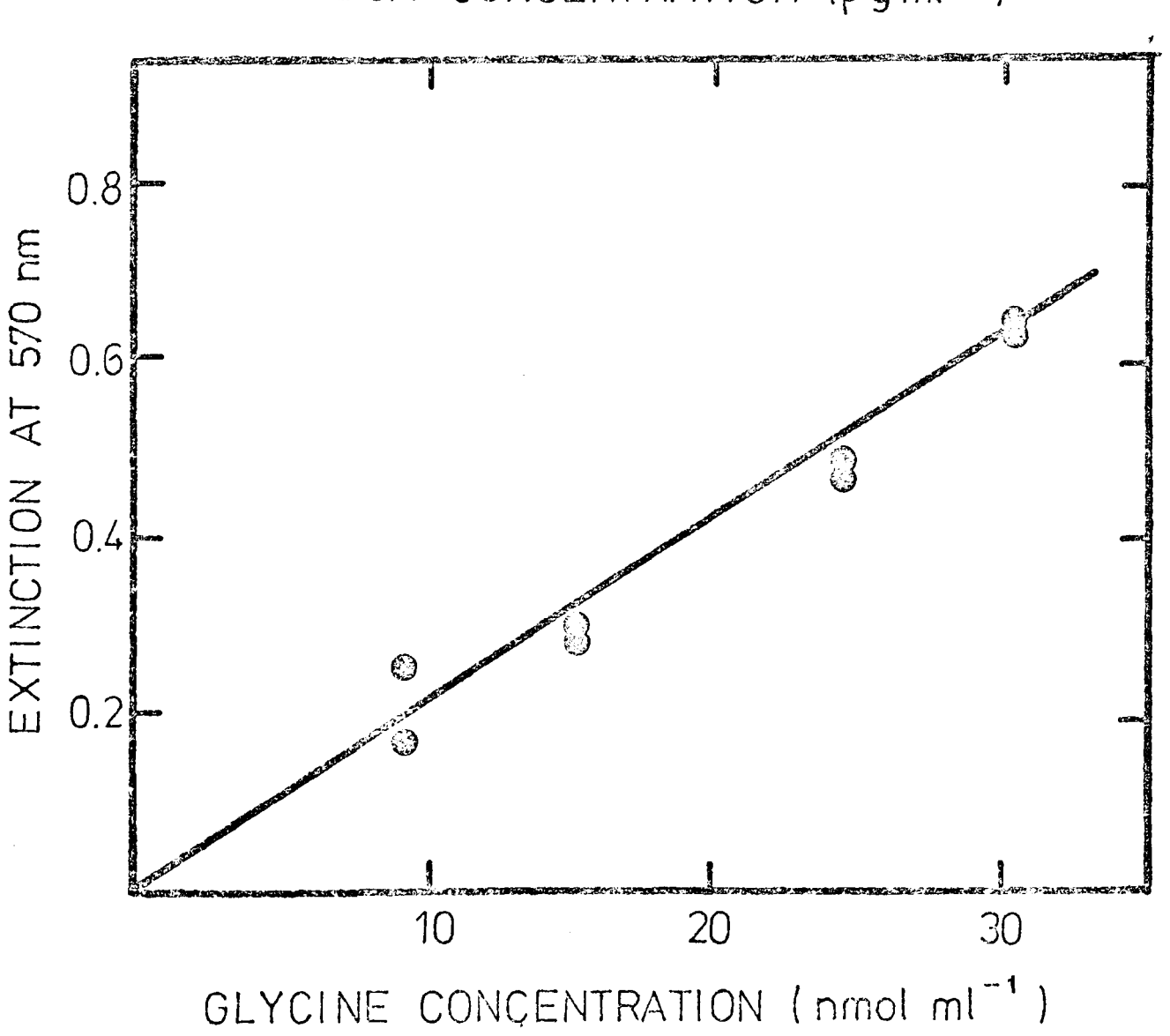

EXTINCTION AT 570 nm
0.8
0.6
0.4
0.2
10
20
30
GLYCINE CONCENTRATION (nmol ml⁻¹)

Figure 2.5 Estimation of Acid Phosphatase activity

Standard used for the estimation of p-Nitrophenol released by acid phosphatase activity assayed as described in the text. p-Nitrophenol given as nmol ml^{-1} in final volume (here = 4.0 ml).

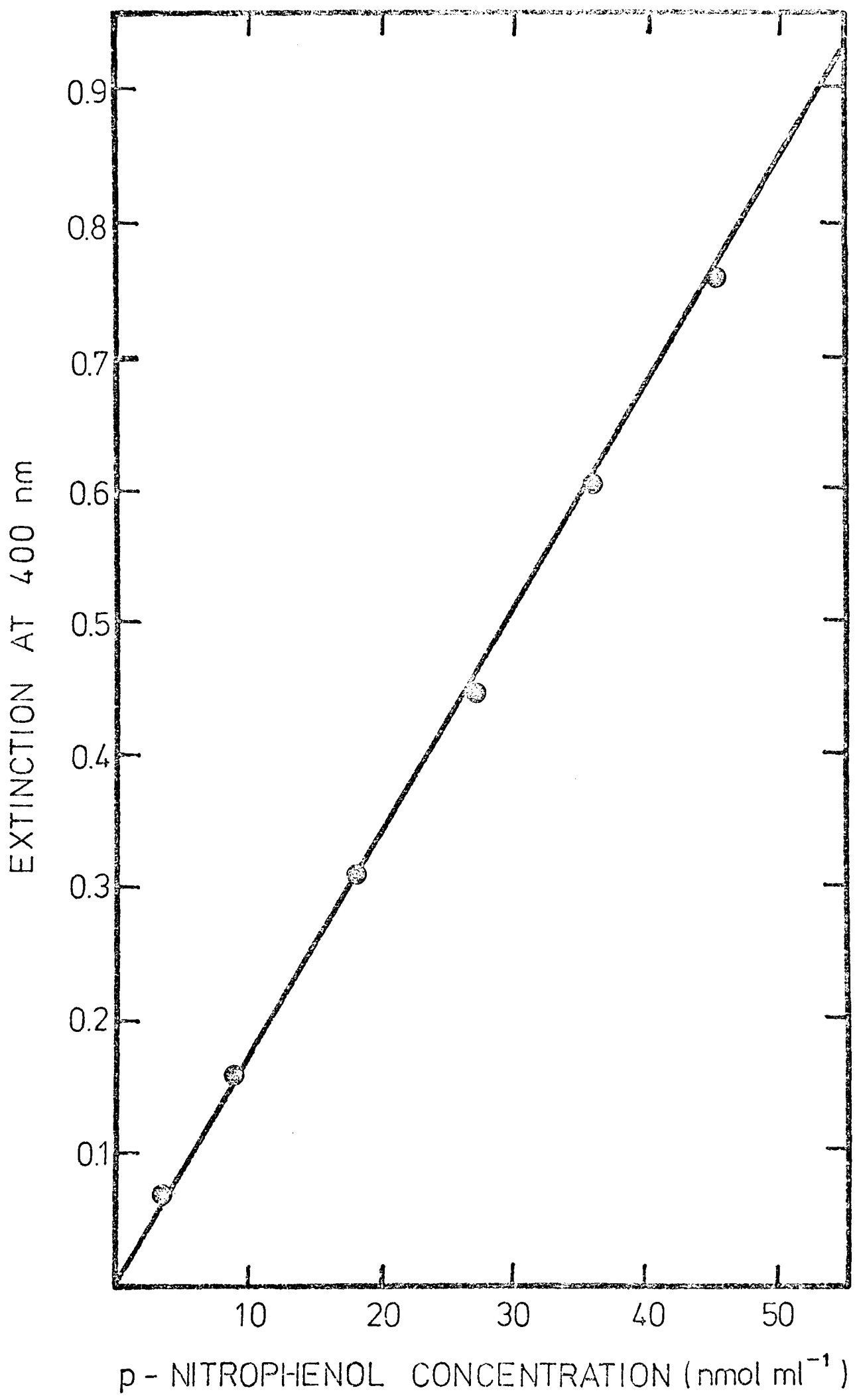
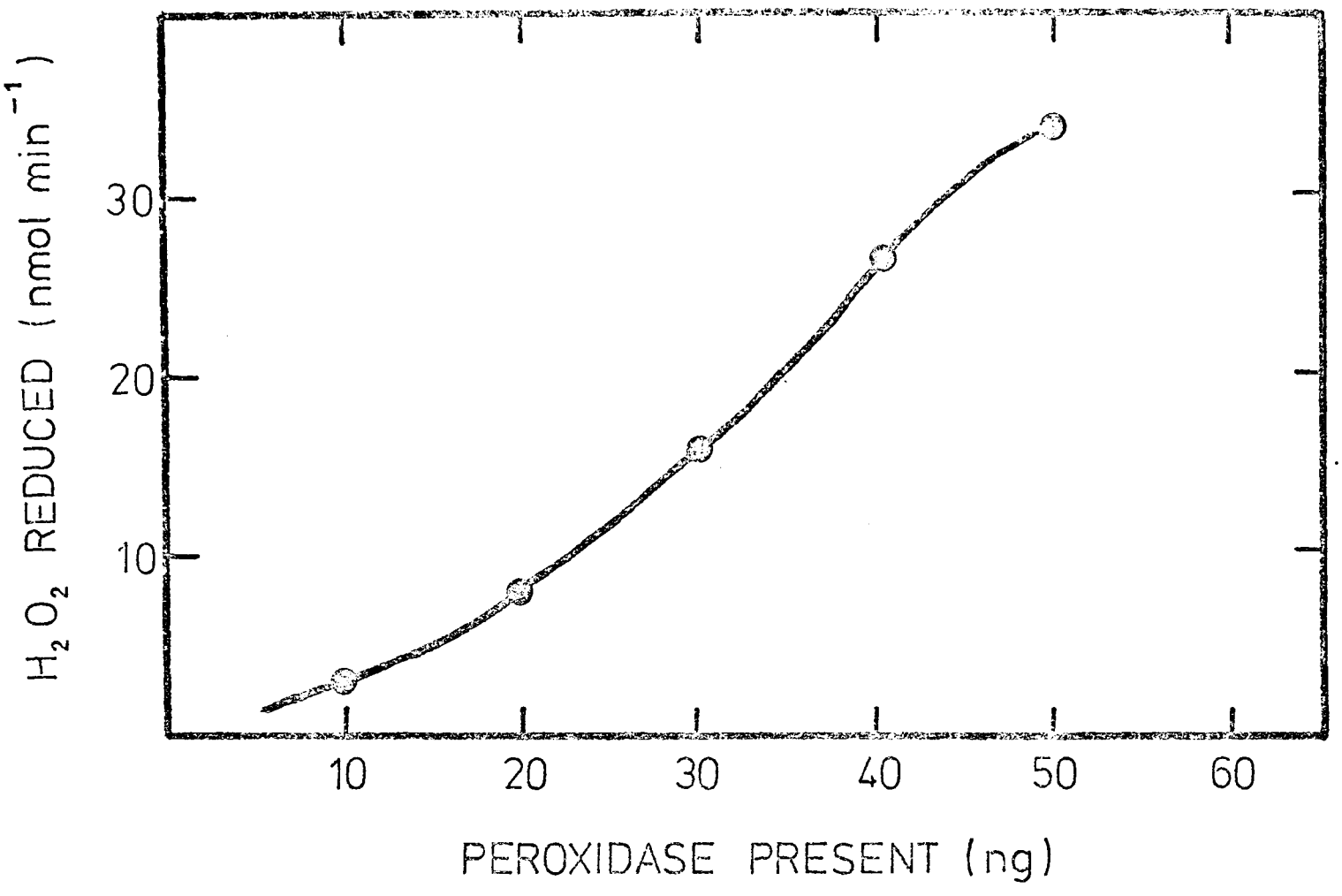

EXTINCTION AT 400 nm
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
10
20
30
40
50
p - NITROPHENOL CONCENTRATION (nmol ml^{-1})

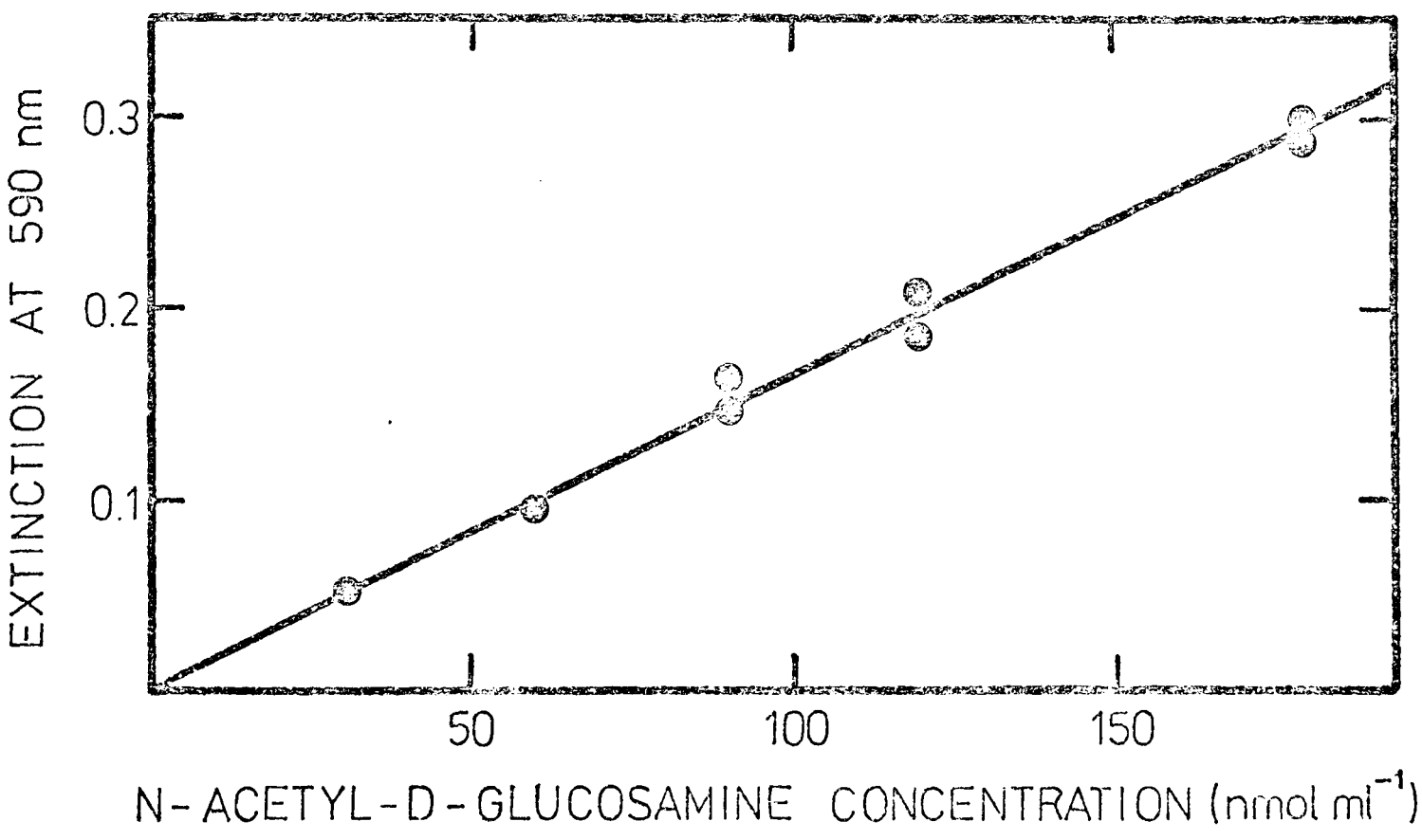
Figure 2.6 Estimation of Peroxidase activity

Samples containing 0-50 ng horseradish peroxidase (SCC) were assayed as described in the text by the method of Chance and Maehly (1955).

Figure 2.7 Estimation of Chitinase activity

An example of a standard used for the estimation of chitinase activity using N-Acetyl-D-glucosamine assayed by the method of Aminoff et al (1952) as described in the text. Line drawn by linear regression. N-Acetyl-D-glucosamine present given as nmol ml^{-1} in final volume (here = 3.1 ml).


H_2O_2 REDUCED (nmol min^{-1})
30
20
10
10
20
30
40
50
60
PEROXIDASE PRESENT (ng)


EXTINCTION AT 590 nm
0.3
0.2
0.1
50
100
150
N-ACETYL-D-GLUCOSAMINE CONCENTRATION (nmol ml^{-1})

CHAPTER 3

THE ULTRASTRUCTURE OF THE DIGESTIVE GLANDS

I INTRODUCTION

II GENERAL FEATURES OF THE RESTING GLAND

1) The Basal Cell

2) The Stalk Cell

3) The Secretory Cell

III A QUANTITATIVE ANALYSIS OF THE CHANGES IN THE GLAND FOLLOWING STIMULATION

IV DISCUSSION

1) General Features

2) Walls

3) The Cytoplasm of the Secretory Cells

4) The Cytoplasm of the Auxiliary Cells

I INTRODUCTION

The ultrastructure of Dionaea digestive glands has been the subject of three previous publications (Scala et al, 1968a, b; Schwab et al, 1969). These satisfactorily established the overall appearance of the cells and, less comprehensively, examined the changes that occur in the glands following stimulation. The digestive glands of other carnivorous plants have attracted some attention, and although a little work has been done with the sessile glands of Pinguicula (Heslop-Harrison and Knox, 1971; Heslop-Harrison, 1975), it is mainly the stalked glands of Drosera (Ragetli et al, 1972; Gilchrist, 1974; Gilchrist and Juniper, 1974), of Drosophyllum (Schnepf, 1960b; 1961a, b; 1963a, b, c) and Pinguicula (Vögel, 1960; Schnepf, 1960a; 1961a; Heslop-Harrison and Knox, 1971; Heslop-Harrison, 1975; 1976) and the quadrifid glands of Utricularia (Vintéjoux, 1973a, b, c; 1974a, b; 1976; Fineran and Lee 1974a, b; 1975) that have been studied.

There is little doubt that the stalked glands of Drosophyllum (Schnepf, 1963a) and Drosera (Rost and Schauer, 1977) secrete a mucilage solution containing negligible protein. Despite this, the ultrastructure of these does not differ appreciably from that of Dionaea, although, as will become apparent, the functional ultrastructural activity in Drosophyllum is very different. In Pinguicula, the stalked glands do contain secretory protein (Heslop-Harrison and Knox, 1971) but are probably primarily concerned with mucin production (Heslop-Harrison, 1975), whereas a much

greater amount of hydrolytic enzymes occurs in the sessile glands (Heslop-Harrison and Knox, 1971). It seems, therefore, that the sessile glands of Drosera and Drosophyllum are likely to be the source of the digestive enzymes in these genera, but this is yet to be investigated.

Ultrastructurally, Dionaea presents a particularly useful system to examine. It is not structurally so complex as to make interpretation difficult; it has a long time-course of secretion; and the gland may be stimulated readily at a known time. Furthermore, as it can repeat a cycle of secretory activity it might be expected that the ultra-structure will return to approximately the same state by the end of the cycle as it was in at the beginning.

II GENERAL FEATURES OF THE RESTING GLAND

The digestive glands which cover the adaxial surface of both lobes of the trap at a density of about 50 mm^{-2}, are exotropic, protruding 20 - 30 μm above the epidermal layer, with a diameter of about 100 μm (Fig. 3.1). They have the appearance of a flattened hemisphere with a domed surface (Figs. 3.2 : 3.3) which itself is allantoid (Fig. 3.4). The glands are semi-sessile and consist of two layers of secretory cells overlying two layers of auxiliary cells, the stalk and basal layers (Fig. 3.5). Each region of the gland has a distinctive ultrastructure, and although the two layers of cap cells are superficially similar there are a number of important differences.

1) The Basal Cell

The basal cells, although continuous with the epidermal cells, are ultrastructurally quite different from them (Fig. 3.6). The normal epidermal cells (Fig. 3.5) are largely devoid of contents but have a mass of cytoplasm in one corner. The basal cells, on the other hand, are filled with numerous spherical vacuoles covering a wide range of sizes and distributed throughout the cell. The majority of these are devoid of contents but some of the smaller ones have a granular matrix, while a few are amorphous, of medium density, and resemble the droplets of the stalk cells (Fig. 3.10). The osmiophilic bodies, noted by Scala et al (1968b), are commonly found both within clear vacuoles and

free in the cytoplasm. The cells have a well-defined nucleus, with a granular matrix and no condensed material. Mitochondria are rare and have poorly developed cristae (Fig. 3.10). A few plastids are present but their morphology is quite distinct from that of the chloroplasts in the adjacent epidermal cells. The latter (Fig. 3.7) show the structures typical of a fully mature C_4-plant chloroplast (Gunning and Steer, 1975) with large starch grains and simple, agranal thylakoids. The plastids of the basal cells are similar to those of the secretory cells (Figs. 3.25 - 3.27).

Endoplasmic reticulum and ribosomes are virtually completely absent, but a few dictyosomes are present and small vesicles derived from these are sometimes encountered apparently fusing with the plasmalemma (Figs. 3.8 : 3.9). The ground-plasm has a dispersed, flocculent appearance, often more concentrated towards the exterior end of the cell (Fig. 3.6). Overall these cells resemble developing tracheids (Clowes and Juniper, 1968) but in the basal cells the tissue is mature, and despite this appearance, shows no connection to the vascular system of the leaf, as is present in Genlisea and Pinguicula (Heslop - Harrison, 1976), Drosera and Drosophyllum (Fenner, 1904), and Nepenthes (Lüttge, 1965).

A striking feature of these cells is the numerous pit-fields between them and all the surrounding cells (Figs. 3.6 : 3.7 : 3.10). There are often several in the walls to the adjacent epidermal cells and at the foot of each basal cell is a pit into the sub-epidermal layer. Both the basal-to-basal cell wall and the basal-to-stalk cell wall contain

large pits, which in the latter may occupy 50 - 75% of the whole cell interface (Fig. 3.6). All these pit-fields are simple and contain numerous plasmodesmata, thus resembling those found elsewhere between living cells, such as in oat-leaf fibres (Gunning and Steer, 1975). Secondary thickening occurs in both walls forming the pits between basal and epidermal cells, while those in the wall to the stalk cell are thickened only to the basal cell side (Fig. 3.10). The pits to the sub-epidermal cells are, however, thickened only at the lower side (Fig. 3.6). Dictyosomes and dictyosomal vesicles are frequently encountered near the regions of secondary thickening (Figs. 3.8 : 3.9), and it seems probable that they are supplying material for building and maintaining these wall ingrowths (Pickett-Heaps, 1967; Robards, 1968).

2) The Stalk Cell

The stalk cell (Fig. 3.10) by contrast contains a large vacuole which is strongly osmiophilic around the edges (Fig. 3.15) and may be highly divided (Fig. 3.10). It closely resembles the vacuoles of the secretory cells (Figs. 3.5 : 3.19). The nucleus is approximately spherical, centrally placed in the cell, contains both condensed and uncondensed fibrillar material, and again resembles those of the secretory cells, rather than the basal cells.

The majority of the cytoplasm (26% cell volume) is filled with spherical droplets, 0.8 - 1.4 μm in diameter and defined in the electron microscope by a sharp single outline (Figs. 3.10 : 3.11). Scala *et al* (1968b) reported these to

be lost from tissue fixed in glutaraldehyde - potassium permanganate and suggested that they were lipid droplets. This was confirmed by treating thin sections with lipase (see 2:II:8). After 90 min treatment, much of the contrast is lost from the droplets (Fig. 3.12), although they still possess a granular matrix. Furthermore, in places the boundary surfaces show a tri-layered structure which resembles a membrane rather than a lipid/water interface.

Fig. 3.13 shows a cell treated for 90 min with lipase. The heavy black deposits present are due to the treatment with lipase and are absent in both the hydrogen peroxide (Fig. 3.14) and untreated (Fig. 3.15) controls. If the tissue is viewed without post-staining (Figs. 4.20 : 4.21) then the deposits are much less intense and it appears that they may be due to the products of the lipase activity taking up heavy metals in the post-staining process. They tend to be concentrated over the endocuticle (the thickened walls to either side of the stalk cells) and the vacuole but the reason for this is not clear.

Mitochondria occupy about 3% of the cell and resemble those in the adjacent secretory cells but have fewer, more disperse cristae. Plastids are uncommon (<1%) and micro-bodies appear to be totally absent. These organelles have been assigned a role in mobilising lipid stores in some tissues (Gruber et al, 1970) but are not always present, as for example in the germination of Phaseolus vulgaris seeds where the lipid stores undergo rapid degradation (Whatley, J. personal communication).

A few dictyosomes and associated vesicles are present while the majority of the endoplasmic reticulum bears ribosomes. All these organelles are, however, less common than in the adjacent secretory cells (Table 3.1).

To either side and between the stalk cells is an area of cell wall staining dark and amorphous in the electron microscope (Fig. 3.16). This region is strongly Sudanophilic (Fig. 3.17), apparently taking up the stain more strongly than the lipid droplets of the stalk cell cytoplasm. The deposit contains suberin, as shown histochemically (Fig. 3.18). It is continuous with the cuticle of the endodermal cells and the thin cuticle of the secretory cells (Fig. 3.16), as noted by previous authors (Scala et al, 1968b). A wall deposit of this nature is a feature common to many exotropic plant glands (Schnepf, 1974) and it is generally accepted that it forms an impermeable barrier, preventing apoplastic exchange between the leaf tissue and the glandular cells (Schnepf, 1974).

3) The Secretory Cell

The most notable feature of the secretory cell (Fig. 3.19) is the extensive vacuolar system which may occupy 50% of the cell volume (Table 3.1). In fact it is composed of several distinct components, each of which varies during the secretory cycle. For analytical purposes therefore, it was subdivided into osmiophilic vacuoles greater than (V_l) or less than (V_s) 2.0 μm in length, and vacuoles with a clear or slightly flocculent appearance (V_c). All types have

an irregular outline and are surrounded by a single limiting tonoplast (Figs. 3.20 : 3.21). The degree of osmiophilic affinity in the former types, and of flocculence in the latter, varies greatly from one vacuole to another, and may even show variation within a single vacuole. Fusion and, presumably, fission of these vacuoles appears to occur readily and frequently small vacuoles are seen partially or entirely enclosed within larger ones. At times, however, the tonoplasts of vacuoles with different staining properties are seen closely juxtaposed indicating there to be some control over the fusion and fission of the sub-components of this cytoplasmic compartment (Figs. 3.20 : 3.21). At these points, concentrations of packed membranes, apparently in a state of storage, sometimes occur (Fig. 3.22).

The nucleus (Fig. 3.23) is approximately spherical with a prominent nucleolus (as noted by Scala *et al*, 1968b). It has some condensed chromatin, occupies 10.5% of the cell volume and is thus very similar to that of the stalk cell. The nuclear envelope has a clear double membrane (Fig. 3.23) with typical nuclear pores (Fig. 3.24) arranged randomly throughout the surface at a density of about 20 μm^{-2}.

Mitochondria may be ellipsoid but often are irregular in profile (Figs. 3.23 : 3.50) with irregularly dispersed cristae. They are numerous, occupying 4.1% of the cell volume and are well dispersed throughout the cytoplasm (Fig. 3.23). Because of the large central vacuole, however, they are often concentrated near to the endoplasmic reticulum and hence near the cell periphery (Fig. 3.27). Scala *et al* (1968b) considered the mitochondria to have 'numerous, well-

defined cristae' but in comparison with their appearance in later stages of the cycle (Figs. 3.51 - 3.53) this is certainly not the case.

A few large plastids are present in each cell profile, occupying 2.1% of the cell volume (Figs. 3.19 : 3.25 : 3.26 : 3.27). They appear to be degenerate (Whatley, J. personal communication), having just a few long tubular thylakoids, numerous plastoglobuli which are often grouped together (Fig. 3.23), and a slightly granular ground-plasm of variable density. This appearance closely fits that described by Scala et al (1968a), who also noted the tubules perpendicular to the thylakoid membranes.

Much of the endoplasmic reticulum, which is common in the secretory cells (Table 3.1), is found lying in long parallel stacks around the periphery of the cell, closely juxtaposed to the plasmalemma (Figs. 3.19 : 3.28 : 3.29 : 3.31). Alternatively, long profiles closely follow the edges of vacuoles, both of the osmiophilic and clear types (Fig. 3.19). Profiles do occur elsewhere throughout the cytoplasm, but much less densely.

Some of the component cisternae of the peripheral stacks are closely associated with desmotubules and long lengths which appear to join to these may be seen (Fig. 3.30). No clear examples of cisternae of endoplasmic reticulum in adjacent cells being joined from one cell to another through the plasmodesmata were found. An association of the endoplasmic reticulum with cell wall protruberances (Figs. 3.28 - 3.31) was noted by Scala et al (1968b), but no explanation was offered for this.

Before stimulation, although much of the endoplasmic reticulum is lined with ribosomes, these tend to be irregularly spaced and widely distributed along the cisternal membranes, while some parts are completely smooth. A few polysomes occur free in the cytoplasm but are seldom seen bound to the endoplasmic reticulum. There are numerous ribosomes free in the cytoplasm (Figs. 3.19 : 3.23).

The appearance of the dictyosomes varies greatly between being apparently totally inactive with the cisternae completely unswollen (Fig. 3.32) to those that appear to be in a state of great activity. These have either large vesicles forming at the maturing face (Fig. 3.33) or the whole cisterna is swollen and totally lost (Fig. 3.34). Although it is possible that this activity has been induced by fixation, it seems likely that the dictyosomes are active when the secretory cells are in the resting state, in contrast to those in Drosophyllum (Schnepf, 1961a). In the inactive state, the dictyosomes have clear intercisternal structures, spaced at about 75 nm apart. Unlike maize root cap cells, however, the intercisternal structure is not visible in active cells (Mollenhauer, 1965).

A notable feature of the walls of the secretory cells is a highly-developed labyrinthine wall, which at times may reach right across the corner of the cell (Fig. 3.35). The internal structure of the projections is continuous with the bulk of the wall and frequently shows a fibrillar central core with a highly electron-transparent region between this and the plasmalemma. This is particularly noticeable in semi-thick sections (Fig. 3.36). Such a structure is

typical of the wall protruberances of many transfer cells (Gunning and Pate, 1969; 1974). Scala et al (1968b) note how these protruberances may extend 2 - 3 x the width of the wall, thus greatly increasing the area of plasmalemma.

Labyrinthine wall protruberances are a common feature of many gland cells and are likely to be an important component in the activity of the Dionaea gland. Fig. 3.37 shows the results of an analysis of the various structural aspects of these wall protruberances. The measure of the extent of increase of labyrinthine wall area is the amplification factor (i.e. the ratio of the length of the plasmalemma to the length of the middle lamella over a distance of cell wall), which shows no correlation to the perimeter length (Fig. 3.37d) and hence must be considered independent of cell size. The amplification factor may, theoretically, be altered by changing either the ingrowth density or the volume of each ingrowth. These two variables are able to be altered without affecting one another as they are independent (Fig. 3.37b). In the outer secretory cells both these factors are positively correlated ($p<0.05$: $p<0.05$) to the amplification factor (Figs. 3.37a : 3.37c) and hence it is probable that they both contribute to the structure of the labyrinthine walls. In the inner secretory cells, however, no correlation was observed between the amplification factor and either of these two variables. Interestingly, both the volume fraction and the ingrowth density show a negative correlation ($p<0.02$: $p<0.001$) to the perimeter length in outer gland cells but only the ingrowth density is correlated ($p<0.001$) in the inner cells, where the amplification factor is much greater (Figs. 3.37e : 3.37f). The

mean ingrowth density, and hence the mean total ingrowths, in a cell of a given perimeter is always greater in an inner gland cell than in an outer gland cell (Figs. 3.37f : 3.37g), although both factors are highly dependent on perimeter length. The amount of labyrinthine wall in the inner and outer secretory cells is one of the most prominent differences between the two cap-cell layers as it is strongly polar, increasing from the outer to the inner side of the gland (Fig. 3.5). The importance and possible significance of this will be considered in detail in Chapter 7.

All the walls between gland cells contain plasmodesmata. These are almost always simple, having an unbranched channel with no Mittelknoten present (Figs. 3.38 : 3.39), but a desmotubule is normally visible in median sections. This may be closely associated with the endoplasmic reticulum (Fig. 3.30). Occasionally, branched examples are found (Fig. 3.40), particularly between the inner secretory cells and the stalk cells. These also show no enlargement of the channel within the wall. In all cases, the plasmodesmata occur in groups containing 5 - 10 channels within a short length of cell wall. In the basal cells, these are enclosed within simple pits (Figs. 3.6 : 3.7), as mentioned earlier. In the walls between secretory cells and the inner secretory cell/stalk cell wall, the wall is usually narrower at these points with no labyrinthine protruberances between the plasmodesmata (Figs. 3.19 : 3.30 : 3.35). Hence, although these regions are not pits in the full sense, they are regions of wall where thickening has been prevented.

The distribution of plasmodesmata within the gland is not random but, like the labyrinthine protruberances, shows a strongly polar arrangement, increasing from the outside to the inside of the gland. This will be considered in detail in Chapter 7.

The exterior wall of the gland is six-layered (Figs. 3.41 : 3.42). At the innermost side is a narrow layer of secondary deposit, continuous with the small labyrinthine protrusions present (1). The bulk of the wall is formed by a lightly-staining fibrillar region, presumably composed of cellulose (2). The third layer is a strip of highly fibrillar, dark-staining material (3), running into the amorphous grey cuticle (4) which has little other internal structure. Beyond the cuticle is a very narrow transparent band (5) and the exterior of the wall is coated with a highly osmiophilic granular deposit, possibly of wax (6). The structure of the epidermal wall is essentially similar but lacking layer (1) and with all the other layers of a very much greater thickness. The cuticle is continuous and neither ectodesmata (Schnepf, 1959) nor cuticular pores (Hall, 1967) have been seen. In section the surface appears smooth apart from the granularity of layer (6), whereas in the scanning microscope it has a more allantoid appearance (Fig. 3.4). This may be due either to much of the wax being removed during fixation for transmission electron microscopy or to partial collapse of the cells during preparation for scanning electron microscopy. Since fresh material viewed in the scanning microscope initially appeared similar to fixed and coated preparations, it seems probable that this

ridging is a real feature of the cell surface.

The wall covering the gland cells is very thin (0.5 - 0.6 μm) compared to that of the adjacent epidermal cell (3 - 5 μm). This is apparently an adaptation for transport into and out of the secretory cells, as might be expected.

III A QUANTITATIVE ANALYSIS OF THE CHANGES IN THE GLAND FOLLOWING STIMULATION

Once the gland cells have been stimulated, they undergo a series of changes in ultrastructure returning finally to approximately the initial state. These changes constitute a true cycle, the glands being capable of reacting to stimulation several further times (Darwin, 1875). In their observation of this cycle, Schwab et al (1969) divided it into five phases of activity based on their interpretation of the ultrastructure. While this helps to clarify the changes that occur, it unnaturally divides a continuous process into a number of discrete actions. Here, therefore, a different approach is used in which the gland structure is quantitatively analysed at regular intervals along the time-course and the changes deduced from interpolation between these points. The overall view is, in fact, not dissimilar, but a number of features require re-interpretation.

Fig. 3.44 shows the results of the Loud (1962) grid-line analysis of the fine-structural changes in the gland cells after stimulation with 4%(w/v) bactopeptone solution. Representative cells from this analysis are shown in Figs. 3.45, 3.46, and 3.47.

One of the most dramatic changes is in the volume of the large osmiophilic vacuoles which decreases by nearly 50% ($p<0.001$) during the first two days but subsequently shows little change, although the small increase between days 4 and 6 is significant ($p<0.1$). Corresponding to the initial

decrease, a large increase in clear or flocculent vacuoles of 3½ x ($p<0.01$) occurs, although this compartment then shows no further significant alteration. The small osmiophilic vacuoles, however, only show a significant increase ($p<0.01$) over the resting state by day 4, although Schwab et al (1969) considered an increase in the number of these to be an early feature of the cycle, coincident with the increase in clear vacuoles.

The ground-plasm volume has increased considerably by day 2 ($p<0.001$) and falls again between days 4 and 6 to its original value. Schwab et al (1969) considered that the poor definition obtained in some cells was due to the state of the cycle, but it also occurs in unstimulated cells (Fig. 3.48). Hence it is more likely to be a fixation artefact. Poor resolution, with a densely-staining ground-plasm, is probably due to the release of easily oxidised material (in this case probably anthocyanin) from the vacuole. In other material this has been avoided by adding caffeine to the fixation medium (Mueller and Rodehorst, 1977). Such treatment was not used here as it might affect the way in which the structural features alter in appearance during the secretory cycle. In well-fixed cells, the ground-plasm remains transparent with a finely granular appearance.

The nucleus by day 2 has undergone a decrease in volume of 27.8% ($p<0.1$), and appears considerably more condensed, while the envelope has become more convoluted. This is still the state at day 4, but by day 6 the volume and appearance have returned to that of the resting cell.

By the second day, the total volume occupied by

mitochondria has significantly increased ($p<0.001$) by x 1.5 (Figs. 3.44 : 3.50-3.53) and, as there is no significant change in the mean volume of each mitochondrion (Table 3.2), this must be due to a real increase in the total number of individuals. Furthermore, this is accompanied by a significant ($p<0.001$) increase in the density of cristae within each mitochondrion (Fig. 3.49a) from 5.8 $\mu m\ \mu m^{-2}$ to 9.0 $\mu m\ \mu m^{-2}$, or by 55.2% ($p<0.001$). Between days 2 and 4 there is no further change, but by day 6 the total volume has returned to normal (Fig. 3.44) although the cristae density is still slightly elevated (Fig. 3.49a). The ratio of cristae length to perimeter length (Fig. 3.49b) closely follows the same pattern but the volume-to-volume ratio (Fig. 3.49c) does not decrease at day 6, indicating that although the total surface area of cristae is decreasing the ones that remain are still swollen (Figs. 3.50 - 3.53). Öpik (1968a) has suggested that swelling in the cristae of mitochondria may be a fixation artefact, but all the examples examined here were treated identically.

During the most active part of the cycle, days 2 and 4, the mitochondria often adopt curious shapes (Figs. 3.54 : 3.55), the elliptical cross-section seen in many cells often being replaced by a much more irregular outline. Frequently they become extremely elongated, with two swollen regions of cristae connected by a long length of narrow reticulum, in which the double membrane is still visible but cristae are absent. The connecting region may stretch many times the length of the swollen termini, and in some sections a ring is seen, indicating a section through a cup-shaped

structure (Fig. 3.46).

The plastids appear to be little affected by stimulation, occupying a constant volume throughout most of the cycle (Fig. 3.44) and being slightly elevated ($p<0.01$) only at day 4. Schwab et al (1969) described the internal structure of these as undergoing alterations parallel to the cycle, with the osmiophilic globules disappearing and a decrease in the extent of the tubular complex. The former alteration was not noticed here, with plastoglobuli being found throughout the cycle (Fig. 3.26). The extent of the tubular complex did not seem to decrease either, there being very variable amounts at all stages throughout the cycle.

Surprisingly, the extent of endoplasmic reticulum per μm^2 (Fig. 3.44) shows a decrease ($p<0.01$) after four days of stimulation (the difference between 0 and 2 not being significant), as does the area of reticulum per μm^3 cell volume. By day 2 however, the appearance of the reticulum has considerably altered with much greater numbers of ribosomes present and far fewer profiles of smooth membrane (Figs. 3.56 : 3.57). Throughout the cycle the majority of reticulum remains close to the plasmalemma, although there is a greater amount elsewhere in the cell than in the unstimulated state. In particular, the profiles around the edges of vacuoles increase with stacks of membrane occurring here (Fig. 3.59). A greater association with dictyosomes is not apparent but the reticulum near the plasmalemma is frequently found in extreme juxtaposition and sometimes apparently fused to the cell membrane at points of invagination (Figs. 3.58 : 3.60). This also occurs in unstimulated cells but to a lesser extent (Figs. 3.28 - 3.31).

Profiles lying extremely close to the plasmalemma are often seen with ribosomes only on the cytoplasmic side and a smooth profile towards the cell exterior (Figs. 3.61 : 3.62). This latter feature is not present in unstimulated cells. As well as an increase in reticulum-bound ribosomes, polysomes appear at day 2 and remain throughout the cycle (Figs. 3.63 : 3.64). These usually appear attached to reticulum membrane in profile and often they are against a darker area of cytoplasm than the rest, as with the nuclear pores in Fig. 3.24, indicating that they are overlying a glancing section of membrane (Bonnett and Newcomb, 1965). Thus it is likely that a large number of polysomes are associated with the endoplasmic reticulum in the stimulated state.

In the resting state, the endoplasmic reticulum cisternae sometimes show a slightly distended profile (Figs. 3.28 : 3.29) but this is not common, and where it occurs the membrane is frequently, but not always, devoid of ribosomes. After stimulation, however, large swellings of the cisternae become much more common, so that in some areas all the profiles are greatly distended (Figs. 3.65 - 3.67). In addition, the membranes have a dense population of bound ribosomes, although polysomes are not found in this situation. At day 4, when these profiles are most prominent, they are also seen to be vesiculating, the vesicle membranes being largely devoid of ribosomes (Fig. 3.65). Alternatively, these are dictyosomal vesicles fusing to the endoplasmic reticulum, or even vesicles formed by reverse pinocytosis at the plasmalemma. The latter alternative seems unlikely, however, in view of the scarcity of reports of this

phenomenon occurring naturally in plant cells.

The volume percent of dictyosomes shows a significant decrease ($p<0.05$) by day 2 which is not reversed by day 6. There is no particular change in their degree of activity and no significant change in the numbers of dictyosomal vesicles present. By day 2 however, some vesicles in the inner gland cells have a rather granular core (Figs. 3.68 : 3.69) while others have a more fibrillar, less dense matrix. Both types may be seen apparently originating from the same dictyosome (Fig. 3.68) and fusing to the plasmalemma. When stained with potassium permanganate, the cisternae of the dictyosomes show a strongly enhanced density (Fig. 3.70) while both the vesicle membranes and their contents do not, implying that they are not lipoidal.

In addition to this increase in wall-building by dictyosomal vesicles, paramural bodies, organelles demonstrated to be associated with wall synthesis (Cox and Juniper, 1973), become more frequent after stimulation. At times they appear free in the cytoplasm close to the plasmalemma (Fig. 3.71) but commonly are present as a collection of vesicles underlying the plasmalemma (Figs. 3.61 : 3.72) in a wall inswelling. A comparison of the dimensions of the membranes (Figs. 3.71 : 3.72) implies these paramural bodies to be lomasomes, rather than plasmalemmasomes (Marchant and Robards, 1968) and suggests that they may have a cytoplasmic origin.

Although the Loud grid-line analysis (Loud, 1962) shows a steady increase in the labyrinthine wall deposit over the

first four days (Fig. 3.44) this is not reflected in the amplification factors along the various wall interfaces (Fig. 3.73) of which the majority show little change over the six days examined. The only significant increases in the amplification factor of the inner secretory cells are seen over the first four days at the interface to the outer secretory cells ($p<0.01$) and at the exterior wall ($p<0.01$). A decrease is seen in the outer secretory cell exterior boundary between four to six days ($p<0.01$) and also in the cell wall between outer secretory cells ($p<0.01$) at this stage. Thus the amplification factors at the majority of the interfaces within the gland cannot be considered to undergo structural alteration as a consequence of stimulation. Because there is so little significant alteration in the labyrinthine walls, the polarity of the gland, which will be considered in Chapter 7, shows no stimulation-induced changes.

No quantitative analysis of the changes in the contents of the stalk cell has been conducted. The lipid droplets do not disappear during the cycle but their contents show signs of depletion. This may either appear as an overall decrease in density of the droplet (Fig. 3.74) or, more commonly, as a clear region in the centre of the droplet (Figs. 3.75 : 3.76). This latter appearance is not an artefact induced by thin-section staining as it is consistent through serial sections. The thin, electron-dense boundary of the droplets remains intact.

IV DISCUSSION

1) General Features

The digestive glands of Dionaea muscipula have the typical ultrastructure of an exotropic plant gland secreting hydrophilic substances and exhibit dynamic ultrastructural changes directly related to this secretory process. Such a structure - with a cap of secretory cells, a stalk with cutinized or suberized walls and a basal cell embedded in the epidermal layer - is common to a wide range of species (Schnepf, 1974).

The morphology of the leaf surface of 12 of the 15 genera of carnivorous plants has been examined, and in all cases glands are present. Pinguicula, Drosera and Drosophyllum all have both stalked and sessile glands which are intermingled over the leaf surface and appear to differ in function. Although both glands in Pinguicula contain hydrolytic enzymes (Heslop-Harrison and Knox, 1971), it is probable that the stalked glands are primarily concerned with the capture of prey and the sessile glands are the primary source of the digestive enzymes. In addition to the digestive glands, Dionaea has small glands around the periphery of the lobes which may secrete polysaccharide, although there is little evidence for this. The two gland-types are spatially separated. In the pitcher plants Nepenthes, Sarracenia and Cephalotus there appear to be several types of gland, spatially separated within the pitchers, but none of these protrude from the leaf surface (Lloyd, 1942).

The glands vary in complexity from a few cells, as in

the quadrifid glands of Utricularia (Vintéjoux, 1974a; Fineran and Lee, 1975) and the sessile glands of Pinguicula (Heslop-Harrison and Knox, 1971), through Dionaea and Drosophyllum with 40 - 50 cells, to the large stalked glands of Drosera containing several hundred cells (Ragetli et al, 1972; Gilchrist, 1974). In all cases, the glands are multicellular and show the same basic structure as Dionaea. A major distinction, however, is that, unlike Drosera and Drosophyllum (Fenner, 1904) with which it is taxonomically grouped, Dionaea does not show any direct connection between the digestive glands and the vascular tissue of the leaf. Neither is this due to this gland being structurally less complex than those of the other two genera as the simple sessile glands of Pinguicula and Genlisea have tracheid vessels immediately below them (Heslop-Harrison, 1976).

2) Walls

In all the carnivorous plant glands so far examined there is an endocuticle which is continuous with the cuticle of the epidermis (Schnepf, 1974; Fineran and Lee, 1975; Heslop-Harrison, 1975; 1976). Most of the glands have a very thin cuticle over the secretory cells, although this appears to be absent in the stalked glands of Pinguicula (Heslop-Harrison, 1975). In Drosera the bell-shaped endodermal layer is fused with the cuticle where they meet, and is thus continuous here also (Heslop-Harrison, 1976), as appears to be the case in Drosophyllum (Schnepf, 1963a). In Dionaea the cuticle is about 175 nm thick, covers the entire surface of the gland and is clearly continuous with the

endodermis, as is the case with the sessile glands of Pinguicula (Heslop-Harrison, 1975) and Utricularia (Fineran and Lee, 1975).

For some time there has been speculation as to whether the cuticles of these glands contain pores through which the secretion product could pass. In Dionaea, as in Utricularia (Fineran and Lee, 1975), the cuticle is quite clearly continuous with no visible pores. In Drosera, however, Ragetli et al (1972) illustrate pores up to 318 nm in diameter in a cuticle which, at 78 nm, is only about 30% as thick as that of Dionaea. Neither does the cuticle of Drosera stalked glands appear as compact as that of Dionaea (compare Ragetli et al, 1972, Fig. 12, with Fig. 3.41). Indeed Heslop-Harrison (1975) has shown that the cuticularized layer may not be continuous, as it becomes detached from the gland when the outer secretory wall is digested with cellulase. Although Schnepf (1961a) failed to find pores in the cuticle of Drosera he did consider that the poorly-defined discontinuities he detected in Drosophyllum (Schnepf, 1960b) were probably pores. In Nepenthes the cuticle does not appear to have pores but, like that of Drosera, is poorly agglutinated (Schnepf, 1965b).

Possibly these pores are only required in those species which secrete large amounts of polysaccharide. Pinguicula, Drosera and Drosophyllum all capture insects with adhesive polysaccharide, which in the last genus Schnepf has amply demonstrated to be produced in the gland cells (Schnepf, 1961b; 1963a, b, c). The solid matter of the secretion of Drosophyllum contains about 0.19%(w/v) polysaccharide

(Schnepf, 1963a) while that of Drosera is about 4%(w/v) acidic mucin (Rost and Schauer, 1977). Neither secretion contains detectable amounts of protein, unlike Dionaea secretion which contains less than 0.01%(w/v) polysaccharide and 1 - 2%(w/v) protein. Thus those glands of carnivorous species that produce polysaccharides have either a thin cuticle containing large pores (Drosophyllum and Drosera) or no cuticle at all (stalked glands of Pinguicula), whereas those that only release protein (Dionaea and the sessile glands of Pinguicula) have a thicker cuticle from which pores are noticeably absent. Certainly in several other species that secrete mucilage, such as Rheum and Rumex (Schnepf, 1968), pores are present, whereas they are absent from the protein-secreting glands of Mercurialis annua (Figier, 1968b; 1969).

In other glands, notably nectaries, several mechanisms appear to be present. In Abutilon (Findlay and Mercer, 1971b) poorly-defined regions of the cuticle are present which they call pores. They propose a mechanism whereby these are 'unplugged' by the hydrostatic pressure of fluid collecting below the cuticle and, if this is so, then it cannot be considered as secretion via pores but rather as an extremely organised form of random cuticular rupture, such as occurs in several other nectaries (Schnepf, 1969), and some salt glands (Thomson, 1975), and as Hall (1967) considers may occur in wax secretion. In some cases, however, the nectar apparently diffuses through the cuticle, as with the secretion of Dionaea. Possibly the mechanism involved depends on the final osmolarity of the secreted solution. In

Abutilon the sugar content is high (Findlay and Mercer, 1971a) and hence the osmotic pressure will be great, whereas in Cicer (Schnepf, 1965a) the secretion contains very little sugar and true cuticular pores are present. Only an examination of a wide range of glands might help to solve this problem. The differences in cuticular structure between Drosera, Drosophyllum and Dionaea may indicate different secretory mechanisms, and this will be considered further in Chapter 5. A mechanism in which cuticular rupture occurs would clearly be detrimental to a gland such as these in which secretion occurs at discrete periods over several weeks.

The other dominant feature of secretory cell walls is the labyrinthine protrusions. These certainly occur in Drosera (Gilchrist, 1974; Ragetli et al, 1972), Drosophyllum (Schnepf, 1960b), Pinguicula (Heslop-Harrison and Knox, 1971; Heslop-Harrison, 1975; Schnepf, 1961a; Vögel, 1960), Utricularia (Fineran and Lee, 1975; Vintéjoux, 1974) and Dionaea (Scala et al, 1968b; Schwab et al, 1969) and probably in the other genera also (Heslop-Harrison, 1975). This feature, initially observed in cells involved in the short distance movement of solutes by Wooding and Northcote (1965), has become widely recognised as an important feature of cells in situations where a rapid exchange of metabolites between the apoplast and the symplast is required.

It was considered by Scala et al (1969) that the labyrinthine walls actually regress during the secretory activity of the cell but in fact this is not the case. Fig. 3.44 shows that the area of labyrinthine wall doubles over

the first four days of the cycle, although there is only a slight change in the amplification factors along the various walls (Fig. 3.73). Thus the significant increase ($p<0.01$) in volume of this wall during the cycle must be due to a swelling of the overall bulk of the wall rather than to an elongation of the labyrinthine protruberances.

Labyrinthine walls occur in situations where either secretion or absorption may be occurring and are seldom evenly distributed within the cells. Thus they line the external walls of the epidermal cells of a number of aquatic species such as Elodea (Falk and Sitte, 1963; Sitte, 1963), Hydrilla and Vallisneria (Gunning and Pate, 1969) which absorb nutrients directly from their environment. The unicellular nectary trichomes of Lonicera contain a dense mass of labyrinthine wall along the external side of the cell (Fahn and Rachmilevitz, 1970) as do the extra-floral nectaries of Vicia (Figier, 1968a). Salt glands are also found with labyrinthine walls along the exterior surface, as for example in Frankenia (Thomson, 1975). In Pinguicula, however, the labyrinthine walls are more elaborate along the internal rather than the external walls of the sessile gland cells and are completely absent from the external walls of the stalked gland cell (Heslop-Harrison, 1975). In Utricularia they are absent from the gland head and occur only along the boundary wall between the basal and stalk cells (Fineran and Lee, 1974a; 1975). Similarly in several hydrathodes and nectaries (Pate and Gunning, 1972) and some salt glands such as Spartina (Thomson, 1975) they occur only along internal walls but in some secretory systems,

such as the trichomes of Pharbitis nil, there are no labyrinthine walls (Unzelman and Healey, 1974). Thus they cannot be considered a prerequisite of exocrine solute exchange systems.

Neither is it possible to establish the direction of flow of solutes from the position of the labyrinthine walls. In Drosera and Drosophyllum they occur on all sides of the secretory cells in both layers of the gland, as in Dionaea. The polarity of the labyrinthine walls which occurs in Dionaea also appears to be present in Drosera although no quantitative data are available. The possible significance of this in terms of the activity of the gland will be considered in Chapter 7.

The internal structure of the labyrinthine walls is clearly closely comparable to that of Pinguicula (Heslop-Harrison and Knox, 1971; Heslop-Harrison, 1975), Drosera (Gilchrist, 1974; Heslop-Harrison, 1975), Utricularia Fineran and Lee, 1975) and Nepenthes (Vassilyev, 1977). Indeed the wall protruberances of transfer cells from a wide range of taxonomic and morphological situations all display this structure (Gunning and Pate, 1969; Pate and Gunning, 1972; Gunning and Pate, 1974). From the staining properties of these walls with the periodic acid - Schiff reaction, alcian blue and toluidine blue, and particularly the effect of pH on these reactions, it has been suggested (Gunning and Pate, 1974) that the core-structure of the wall is composed of carbohydrates in which numerous carboxyl groups are available for methylation. The clear region shows no reaction to callose stain and these authors suggest that it

may in fact be due to shrinkage of the fibrillar core material during preparation. It seems unlikely, however, that this is the case in Dionaea as the outline of the plasmalemma often bears little relationship to the positions of the core-matrices. Leaching of material from this region during preparation may be occurring, since in some cases (e.g. Fig. 3.35) there is no clear region between the core and the plasmalemma.

The labyrinthine walls are laid down very late in the development of the gland (see Fig. 4.25), within the last few days of maturation. The polarity of the positioning of the protruberances within the cell shows there to be a precise control over this activity in Dionaea, as elsewhere (Gunning and Pate, 1974). In leaves (Gunning et al, 1968) and minor veins (Pate et al, 1970), transfer cells do not develop if the plants are kept in conditions of low light or solute or carbon dioxide depletion, although this is reversed by restoring these factors. It would be interesting to see whether Dionaea is influenced by environmental factors or, as a mature plant forming transfer cells, is programmed to lay them down in the last stages of development. They are apparently not induced by the presence of absorbed solutes, as may be the case in seedlings (Pate et al, 1970), as the major part of the wall is present before absorption begins. Fahn and Rachmilevitz (1970) find the labyrinthine wall deposition to start early in the development of Lonicera nectaries, showing that it is not always a late event.

The factors which control the deposition of these labyrinthine deposits may be indirectly examined by studying

the architecture of the wall protruberances, the results of which are given in Fig. 3.37. These show that some aspects of the architecture of transfer walls in Dionaea glands differ from those of Vicia faba leaf veins (Gunning et al, 1974). In both tissues the cell perimeter is positively correlated with the total number of ingrowths. The cell sizes in Dionaea are much greater (33 - 79 μm) than in Vicia (3 - 49 μm) but the shape of the correlation is very similar. In Dionaea glands, the cells are able to maintain a high number of ingrowths as they get smaller by increasing the density at which the ingrowths are laid down. Although there must be a limit to the extent to which this can be done, it is not reached in this system. Thus, in inner gland cells, by increasing the density of ingrowths, the volume of cell occupied by cell wall (and hence the extent of increase in the area of the plasmalemma) is made independent of cell size. This is confirmed by the fact that the volume fraction and amplification factor show no correlation.

In the outer gland cells, however, the perimeter is negatively correlated to the cell wall volume, as it is in Vicia, but in Vicia veins the number of ingrowths per μm of perimeter shows no correlation to the amplification factor, whereas these parameters are correlated in Dionaea outer secretory cells.

Thus the architecture of the labyrinthine walls in the secretory cells of Dionaea differs not only from one layer to another but also from that found in the leaf vein of Vicia. In the inner secretory cells, the control over deposition favours a

sufficiently high ingrowth density to make both the surface area and the volume of the ingrowths independent of the length of the cell perimeter. In the outer secretory cells, where the ingrowth density is lower for a given perimeter, then the volume fraction of wall has to be increased in the smaller cells to maintain a constant amplification factor. The importance of maintaining a high amplification factor, particularly in the inner secretory cells, will be considered in Chapter 7.

Lomasomes, which occur quite commonly in some of the secretory gland cells may be involved with the synthesis of labyrinthine cell wall, as it has been suggested by auto-radiographic labelling that they have an important role in the synthesis of non-cellulosic cell wall (Cox and Juniper, 1973) and of the chitinaceous wall in fungi (Marchant and Robards, 1968). Coulomb (1973) shows lomasomes and multi-vesicular bodies in Scorzonera which have an appearance very like those in Dionaea and stain positively for acidic polysaccharides. It has been suggested (Marchant and Robards, 1968) that their role is in providing centres of synthetic activity and that the lomasomes are actually involved with the movement of cell-wall synthesising enzymes across the plasmalemma. Such a role is compatible with the observed distribution of lomasomes in Dionaea, where they are closely associated with regions of labyrinthine-wall growth. Marchant et al (1967) consider the lomasomes in Phycomyces to be originating from the endoplasmic reticulum but there is no evidence for this in Dionaea.

The plasmodesmata which occur throughout the digestive

glands of Dionaea present an alternative mechanism for the inter-cellular exchange of solutes (Spanswick, 1974) to the route via the apoplast, utilising the labyrinthine wall protruberances to enter the cells. These inter-cellular channels have been the subject of much research (Gunning and Robards, 1976). The channels in Dionaea are simple, containing a constricted desmotubule and, when viewed in true median secretion (Fig. 3.39), show no constriction at the termini. The internal diameter varies considerably from one boundary wall within the gland to another (Table 3.3) but they all fall within the established range of sizes (30 - 90 μm) obtained in other tissues (Robards, 1976), although mostly in the upper region. Unfortunately, very few high resolution photographs of plasmodesmata from secretory cells are available, but Table 3.3 shows some data from where these could be obtained. In most cases, the dimensions are comparable to Dionaea and possibly particularly large plasmodesmata are a functional feature of glandular cells.

The structure of the plasmodesmata in Dionaea bears some similarity to those between the mesophyll and bundle sheath cells in C_4 - plants, such as sugar cane and Aristida ascensionis (Osmond and Smith, 1976) and wheat and oats (O'Brien and Carr, 1970), all of which have linear channels with a narrow, clear desmotubule, although in these species the plasmodesmata are constricted at both termini. Those in Drosera (Ragetli et al, 1972) are similar both in appearance and size to the plasmodesmata in Dionaea.

Furthermore, in all these cases the plasmodesmata are densely confined to small regions of the wall, apparently

being excluded from the others by secondary thickening. In sugar cane, wheat and oats, the wall is suberized and the plasmodesmata are in pits, making this wall closely resemble the stalk cell/basal cell wall of Dionaea. In Drosera and Pinguicula the secretory cell walls have groups of plasmodesmata arranged in regions of wall showing minimal thickening, while the endodermal cell/stalk cell wall in Drosera (equivalent to the stalk cell/basal cell wall of Dionaea) has thickening to both sides of the pits like the basal cell/epidermal cell walls of Dionaea.

Hence it appears that this arrangement of plasmodesmata is favoured in other situations where a rapid exchange of metabolites is desirable. Indeed there are strong similarities with the companion cells loading and unloading phloem which may, as for example in Senecio vulgaris (Gunning and Pate, 1974), use both labyrinthine walls and plasmodesmata to fulfil their role. In this comparison, the xylem elements are equivalent to the exterior of the leaf: the sieve elements to the stalk cells. In some cases such as Pisum and Lupinus (Gunning et al, 1968), however, although the arrangement of plasmodesmata within the transfer cells closely resembles that in Dionaea glands, they are found within a slightly distended part of the cell wall, not a contracted region. Whether there is any significance in this is not clear at present.

In Utricularia (Vintéjoux, 1974a; Fineran and Lee, 1975), however, the plasmodesmata in the quadrifid glands are evenly distributed along the walls and frequently compound. Whether this is a reflection of a different function is not clear, but

it seems likely that these glands are involved in the production of digestive enzymes, at least in some species (Vintéjoux, 1974a, b). Since these glands are always in an aqueous environment it may be that the secretory mechanism functions better with fewer plasmodesmata, perhaps due to a different water balance in the cells. The possible role of the plasmodesmata in Dionaea will be considered in Chapter 7.

3) The Cytoplasm of the Secretory Cells

The large osmiophilic vacuoles so dominant in the cytoplasm of Dionaea secretory cells are also prominent in the secretory cells of the stalked glands of Drosera when fixed in 100 mM-sodium cacodylate buffer, pH7.2 (Gilchrist, 1974) but are absent from material prepared in 100 mM-phosphate buffer, pH7.0 - 7.3 (Ragetli et al, 1972). In the latter case, however, they are present in the small glands on the stalk of the tenticle but are apparently entirely absent from the cap-cells. In both cases, however, the vacuoles are extremely prominent but in the former preparation (Gilchrist, 1974) they are more sub-divided, with small osmiophilic vacuoles, making the cells closely resemble Dionaea. Nepenthes (Vassilyev, 1975) similarly has large osmiophilic vacuoles but in Utricularia (Vintéjoux, 1974a, b; Fineran and Lee, 1975) and Drosophyllum (Schnepf, 1963a, b, c) the vacuoles do not appear to be osmiophilic at all but more like the clear vacuoles which become more plentiful in Drosera secretory cells late in the digestive cycle.

Pinguicula appears to be rather variable. Schnepf (1961a; 1963a) found the vacuoles of the stalked glands to be highly osmiophilic and divided, as in Dionaea; Heslop-Harrison (1975) found them to be very variable, possibly due to the discharge of their contents. She shows an electron micrograph of a sessile gland, however, (Heslop-Harrison, 1975, Fig. 2.9) in which the vacuole is full of a dense flocculent material, rather resembling the appearance sometimes presented by Dionaea (Fig. 3.20). It has been suggested in several of these systems that the vacuole is used to store the secretory material (Vögel, 1960; Scala et al, 1968a; Schwab et al, 1969; Heslop-Harrison, 1976). Whether or not this is the case in Dionaea will be discussed in Chapter 4.

Upon stimulation, the vacuolar volume of Dionaea decreases substantially, as noted by Gardiner (1883) and Schwab et al (1969). This is also true of Pinguicula (Heslop-Harrison, 1975) but no change in the form of the vacuoles in Drosophyllum was noted by Schnepf (1974). Schwab et al (1969) also noted the increase in volume of the vacuoles with clear or slightly flocculent contents. This is supported by this quantitative analysis. The complexity of the vacuoles in Dionaea illustrates the problems of interpreting the dynamics of ultrastructural changes in a system where secretion and absorption are occurring simultaneously.

The appearance of the vacuole can be dependent on the nature of the fixation process used (Fineran, 1970) and therefore it is important in a study like this one to ensure

very uniform fixation conditions. Inclusion bodies (Fineran, 1971) are occasionally seen within the vacuoles of Dionaea secretory cells (Fig. 3.19) and folded membraneous structures at the edges (Fig. 3.22). The latter probably act as a store of membrane components, but the function of the former is not clear. Cells with several discrete vacuoles having different staining properties have been reported elsewhere (Gay et al, 1971) but no explanation was tendered here for their roles. Gifford and Steward (1967), however, found cells in Kalanchöe blossfeldiana with both very dark-staining vacuoles and ones with a light, flocculent stain. These latter usually had a darker staining ground-plasm. They considered that the vacuoles were normally filled with anthocyanin and that in the cells with light-staining vacuoles and a dark ground-plasm this had been released due to a rupture in the tonoplast. Thus the situation in Kalanchöe appears very similar to Dionaea where anthocyanin is also stored in the vacuoles (see Chapter 4) and its release has similar effects on the fixation of this tissue.

Gardiner (1883) reported that the nucleus of Dionaea decreases in size after stimulation, and this is supported by the present study. There seem to be no special features of the nucleus to distinguish it from those of other non-dividing plant cells except that it occupies a larger volume of the cell than average. Gunning et al (1974) point this out as a common feature of transfer cells which is due to the total cell volume being below average. After stimulation, however, it appears to be more active with a

more convoluted nuclear envelope, although the nucleoplasm shows little alteration in its appearance. Schnepf (1963c) and earlier workers (Lloyd, 1942) showed there to be rapid and profound changes in the nucleus of Drosophyllum after stimulation, with the chromatin aggregating around the periphery of the nucleus within 30 min. Again this implies that Drosophyllum is responding very differently from Dionaea and that perhaps mucilage secretion is initiated by a nuclear and not a cytoplasmic response.

The density of nuclear pores is quite high (20 μm^{-2}) and probably indicative of an actively synthesizing nucleus (Lafountain and Lafountain, 1973). Fig. 3.24 is from an actively secreting cell and it would be interesting to examine the nature and density of nuclear pores at different stages of the cycle to see whether they alter in response to the increased cellular activity, as found by Lott et al (1972) for Cucurbita colyledons.

Nuclear crystals, such as those found in the stalk cells of Pinguicula (Heslop-Harrison, 1975) and the developing gland-cells of Utricularia (Vintéjoux, 1970a) and common in the trichome cells of Pharbitis nil (Unzelman and Healey, 1972) are not found in Dionaea at all.

The mitochondria of the unstimulated glands are characteristic of plant cells (Gunning and Steer, 1975) and they are present in large numbers in all the carnivorous plant secretory cells examined. High concentrations of mitochondria, occupying up to 20% of the cell volume (Gunning and Steer, 1975), are common in all transfer cells (Gunning and Pate, 1974) and are a very obvious feature of

cells where extremely high levels of transport are occurring (Lüttge, 1971). The mitochondria in Pinguicula (Heslop-Harrison, 1975), Drosera (Gilchrist, 1974), Drosophyllum (Schnepf, 1960b; 1963a, b,c) and Nepenthes (Vassilyev, 1975) are all similar in structure to those in Dionaea. Schnepf (1961a) shows there to be between 23 and 37 mitochondria per 100 μm^2 of gland cell in the stalked and sessile glands of Drosophyllum which is very much higher than the mean number in Dionaea (about 6), even allowing for the contribution of the larger vacuolar compartment to the cell volume (about 9). The figures quoted by Schnepf (1961a) are considerably higher, however, than may be derived from his published photographs (about 12). In Pinguicula "far more" mitochondria (Heslop-Harrison, 1975) are found in the secretory cells than the stalk cells, as in Dionaea (Table 3.1).

It has been shown (Ziegler, 1956; Mercer and Rathgeber, 1962) that a high density of mitochondria in actively secreting glands is correlated with a high respiratory rate in that tissue as compared to adjacent, non-secretory tissue.

Following stimulation, the mitochondria undergo changes in ultrastructure clearly indicative of greatly increased respiratory activity (Hackenbrook, 1966), with a large increase in the cristae area without any increase in the size of the organelle. Similar changes during the development of Arum flowers have been correlated with increased respiratory activity (Simon and Chapman, 1961) but no quantitative data are available and the density of cristae in these cells is very low compared to Dionaea.

Mitochondria in transfer cells and active secretory cells clearly have more densely packed cristae than cells not specifically concerned with transporting metabolites across the plasmalemma (Gunning and Steer, 1975). Schnepf (1963b) shows that the mitochondria in Drosophyllum have a higher cristae density after stimulation.

Comparable quantitative data appear to be unavailable for plant cells but in animals figures of 30 and 90 μm^2 cristae membrane per μm^3 of mitochondrion have been measured in liver and heart cells respectively (Gunning and Steer, 1975). In the stimulated state, Dionaea equates with the higher of these values, which is for a tissue with a large and continuous need for ATP. Schwab et al (1969) commented on this apparent increase in mitochondrial activity, although the mitochondria of their preparations do not display as densely packed cristae as found here.

The contorted shapes adopted by many of the mitochondria in active secretory cells increases the surface/volume ratio of the mitochondria. This may well be essential to obtain a sufficiently rapid exchange of metabolites with the cytoplasm to maintain the high rates of ATP synthesis suggested by the cristae area.

The description of the plastids given by Scala et al (1968a, b) and Schwab et al (1969) leaves little to be added. The sequence of changes that they (Schwab et al, 1969) envisage as occurring after stimulation was not, however, apparent here. Plastids of all the types which they describe appear to be present at all stages of the cycle,

no one type being uniquely associated with any particular phase of secretory activity. The plastids in the secretory cells of Drosera are very similar to those in Dionaea shown in Fig. 3.19 (Schnepf, 1961a; Gilchrist, unpublished micrographs). Heslop-Harrison (1975) describes those in Pinguicula as large and "extended into amoeboid or vermiform processes". While this sounds rather different from those in Dionaea, the micrographs have not yet been published and those of Pinguicula in Vögel (1960) and Schnepf (1961a) show a structure closely related to the plastids in Dionaea. Vögel (1960) shows the plastids sheathed in several layers of endoplasmic reticulum, but this is not present in Schnepf (1961a) and may be an uncommon occurrence. In Drosophyllum there are rather more plastids in the cells of the stalked glands than in the sessile glands, and, although they differ from those of Pinguicula, Drosera and Dionaea in occasionally containing starch grains, the appearance is otherwise very similar (Schnepf, 1961a; 1963a, b) and the number comparable to Dionaea.

The long unstacked thylakoid membranes, the plasto-globuli and the tubular complexes all give the impression that these plastids are in a non-functional state and probably degenerate. In the bean root-tip plastids that they closely resemble (Newcomb, 1967) the tubular complex appears to be involved with synthesising a sac of storage protein. But although the tubular complex in Dionaea is very similar (as noted by Scala et al, 1968a) there is no associated protein sac. Newcomb (1967) suggests that the tubular complex arises from the inner envelope membrane via

the tubules seen throughout the cell (Figs. 3.25 - 3.27). Although there is no evidence for this it could provide an elegant way to withdraw the membranes into a state of storage where the plastid is inactive, as in Dionaea. Some of the tubular complexes are sufficiently organised to resemble the prolamella body of etioplasts (Fig. 3.27) but this tissue is mature and exposed to a full light regime. Thus, like that in the plastids in bean root-tip cells (Newcomb, 1967), the tubular complex is light-insensitive.

Whether these plastids are ontogenetically degenerate or immature remains to be determined. Certainly they are unable to undergo photosynthetic activity and therefore, in the resting state, all the metabolites required for the respiratory and synthetic activities of the gland must be supplied from the photosynthetic leaf tissue via the stalk and basal cells. Once stimulated, however, plentiful metabolites are available by absorption from the exterior, and it is at this stage that these are required.

There is a considerable amount of endoplasmic reticulum in the gland cells (Table 3.1) with a cross-sectional density equivalent to that of the mitochondrial cristae within the mitochondria (Table 3.2). The values for endoplasmic reticulum given in Table 3.1 column ER(1) express the length of membrane sectioned per cross-section of cell ground-plasm and thus make no assumption about the shape of the reticulum. If, however, the cisternae are assumed to be sheets of membrane, as indicated by Fineran (1973), rather than tubules, then an estimate of the area of membrane per unit area of cytoplasm can be obtained (Table 3.1 column

ER(2)). The concentration of reticulum in outer secretory cells at the start of the cycle is thus comparable to the 15 - 20 μm^2 μm^{-3} in pancreatic exocrine cells and the 6 - 12 μm^2 μm^{-3} in tapetal cells of developing Avena pollen grains (Gunning and Steer, 1975), both systems known to be extremely active in protein synthesis. The inner secretory cells possess rather less endoplasmic reticulum than the outer cells but still have a density comparable to the tapetal cells and to liver cells (Gunning and Steer, 1975). The stalk cell is more comparable to ordinary root-tip cells, not considered to be especially active in protein synthesis.

Endoplasmic reticulum has been established beyond reasonable doubt as the key site of protein synthesis in both animal (Caro and Palade, 1964; Siekevitz and Palade, 1960) and plant (Callow and Evans, 1974; Chen and Jones, 1974; Figier, 1969) cells. Furthermore Scheele et al (1978) have recently shown that the biological selection of proteins for secretion or cytoplasmic function is probably controlled at the point of synthesis at the endoplasmic reticulum membrane. Therefore, the activity of this organelle in specialized secretory cells is particularly important.

Most of the structural features seen in the endoplasmic reticulum of Dionaea have been observed elsewhere, but always in actively secreting systems. Swellings in the cisternae of the reticulum were noted by Jensen (1965) who considered them to contain proteinaceous deposits. He also observed a very high ribosomal population. Coulomb et al (1972) observed similar swellings in root cells of Cucurbita, which they considered to be proto-lysosomes. Extensively distended cisternae of endoplasmic reticulum

were found in the root hairs of radish (Bonnett and Newcomb, 1965; 1966) which contained a dense fibrillar matrix. No explanation was tendered as to the role of this.

The ultrastructural changes which occur in the endoplasmic reticulum of Dionaea imply that this organelle makes an important contribution to the secretory activity of the gland. How it is involved will be discussed in Chapters 4 and 5.

The dictyosomes show a structure typical of that for plant cells (Gunning and Steer, 1975), although they are small with the maximum width of a cisterna seldom exceeding 1 μm. The number of cisternae in each dictyosome varies from 4 - 8 and in some cases these show almost no vesiculation at the periphery, apparently being in a completely quiescent state. Only then are the intercisternal elements described by Mollenhauer (1965) visible and which Franke et al (1972) have suggested may act to hold the cisternae together, only allowing vesiculation at the periphery.

Dionaea shows two distinct morphological types of active dictyosome (Figs. 3.33: 3.34). One has the cisternae tightly apposed with large vesicular swellings at the termini. This resembles the dictyosomes in many plants, including Drosophyllum (Schnepf, 1960b; 1961a; 1963a, b, c) and the well-studied root-cap of Zea mays (Clowes and Juniper, 1968; Gunning and Steer, 1975) although the vesicles are even more spherical than in these cases. The other has cisternae evenly swollen right across their entire length and resembling those in root-cap cells of Phleum pratense (Gunning and Steer, 1975) and Triticum vulgare

(Pickett-Heaps and Northcote, 1966).

Even before stimulation there is a condiderable variety in the states of activity of the dictyosomes, and no significant change is induced in this by the initiation of secretory activity. This is in complete contrast to a large number of secretory systems (Lüttge, 1971; Schnepf, 1974) where the dictyosomes appear to play an important role. Schwab et al (1969) considered there to be an increase in dictyosomal activity in the later part of the cycle with more dictyosomal vesicles present. Certainly by day 4 there is an increase in the number of dictyosomal vesicles present in both layers of secretory cells, but this does not persist through to day 6.

Some of the vesicles derived from both of these types of dictyosomes have a granular or fibrillar core, resembling the core material of the labyrinthine cell wall. Fusion between vesicles from dictyosomes and the plasmalemma are not commonly seen but in some cases these vesicles are observed fusing with the plasmalemma in stimulated cells (Figs. 3.68 : 3.69). The contents of the vesicles closely resemble the polysaccharides secreted by many root cap cells (Pickett-Heaps, 1967; Paull and Jones, 1975) suggesting that it may be polysaccharide which is being released. This being so, then most probably the dictyosomes are supplying the material used to build the labyrinthine cell walls, as carbohydrate is an insignificant component of the secretion. Such a system would readily lead to the observed shapes of the labyrinthine walls, which have already been discussed.

The importance of the dictyosomes in the synthesis of

secondary cell wall is well documented. Robards (1968) shows dictyosomal vesicles extensively fusing with the plasmalemma in xylem differentiation while Gunning et al (1968) show vesicles very similar to those in Dionaea fusing to forming protruberances in transfer cells of Pisum. Northcote and Pickett-Heaps (1966) and Pickett-Heaps (1967) have clearly shown that these vesicles carry the precursor material for the biosynthesis of the cell wall in root-cap cells of wheat.

Small vesicles, apparently derived from dictyosomes, are often seen fusing with one another (Fig. 3.33 : 3.65) and possibly some of the small clear vacuoles are derived from this process. As these vacuoles may be involved in the absorptive activity of the gland (see Chapter 7) it is possible that the dictyosomes have a role to play in this activity.

The dictyosomes show no association with profiles of endoplasmic reticulum, as commonly occurs elsewhere, and neither are membrane-bound vesicles observed at the forming face. There is thus no evidence for an endomembrane system in Dionaea cells, as proposed by Morré and Mollenhauer (1974) for a wide number of secretory cells.

It is proposed, therefore, that the dictyosomes in Dionaea are performing two roles. In one, they are the site of synthesis and form the export vesicles for the construction of the labyrinthine cell walls, while in the other they synthesize and supply membrane units for the formation of intracellular vacuoles. Since the labyrinthine wall swells after stimulation but does not become more infolded, the

former role may largely occur in the last stages leading to maturity and thus examples in mature tissue are uncommon. The dictyosomes then may be acting primarily as a source of suitable membrane to be supplied when new vacuoles are made, and the observed decrease in numbers is compatible with the observed increase in clear vacuoles within the secretory cells. This hypothesis might be tested using radiolabelled cell wall precursors and cytochemical methods in thin sections to investigate the movements of the dictyosomal vesicles and the nature of their contents as, for example, in Northcote and Pickett-Heaps (1966).

Both these concepts, of the bifunctional role of dictyosomes and of the dictyosomes supplying membrane units, are not new. Robards (1966) and Pickett-Heaps (1967) both point out that the Golgi are probably performing more than one role in the cell and Ovtracht and Thiery (1972) show that in snail multifid glands each individual cisterna of the Golgi has distinct regions with different staining properties. They demonstrate that the light-staining areas contain polysaccharide while the dark areas are proteinaceous, and that these regions vesiculate separately. In their study of the nectaries of various species of Vinca Rachmilevitz and Fahn (1973) note how the dictyosomes show their greatest activity before the secretory phase and suggest that they are supplying membranes for the increasing amounts of endoplasmic reticulum in these cells as they mature. A similar situation in Dionaea might occur and a developmental examination of the ultrastructure of the

glands is required to examine this. A role for endoplasmic reticulum in the production of various kinds of vacuoles is well documented (Berjak, 1972; Matile, 1974).

4) The Cytoplasm of the Auxiliary Cells

The grey, amorphous bodies in the stalk cells are shown by lipase digestion to be lipid. What type of lipid has not been ascertained, although the lipase used was a triacylglycerol acyl hydrolase (EC 3.1.1.3) and therefore much of the lipid was probably triacylglycerol derivatives. The lack of affinity of these droplets for Sudan III (Schwab et al, 1969) and Sudan IV indicates, however, that they may be more complex than this.

The spherosomes of peanut seeds examined by Jacks et al (1967) closely resemble the lipid bodies of the stalk cells both in size and staining properties with different fixatives. These are shown (Jacks et al, 1967) to be 98% lipid but to contain sufficient protein (1.3%) to have a limiting membrane, which is visible after hexane extraction. After extraction, however, these spherosomes do not show a tri-layered membrane, as in Dionaea. The protein bodies described by Caro and Palade (1964) show similar staining when fixed with osmium tetroxide, but a membrane is visible.

Thus these bodies may be regarded as true spherosomes - sites of lipid storage but not of lipase activity (Jacks et al, 1967). During the digestive cycle there is some loss of contrast from these droplets, but it is seldom complete. Probably they act as a reserve store of energy, only called

upon when insufficient energy is supplied to the gland to maintain the high rates of respiration likely to be associated with the transporting activity of the secretory cells.

Table 3.1: Quantitative Analysis of the Structure of the Gland Cells

Cell	Days After Stimulation	Cytoplasmic Compartment[a]																
		$1^{o}CW$	LW	V_c	V_s	V_l	MVB	N	M	LD	P	D	DV	L	GP	ER(1)[b]	ER(2)[c]	No.[d]
Outer Secretory Cell	0	12.2	2.0	1.9	2.8	41.5	0.38	9.8	3.6	-	1.8	1.4	0.39	0.63	21.6	8.18	13.25	5(3)
	2	13.8	3.4	8.6	4.2	22.2	-	7.0	9.0	0.1	1.4	0.61	0.62	-	29.1	9.60	14.14	5(3)
	4	13.0	4.2	7.4	6.3	20.7	0.24	5.9	7.3	0.68	2.7	0.73	1.1	-	29.8	6.05	5.76	7(3)
	6	12.0	2.6	11.1	6.2	28.4	-	10.0	4.9	-	1.7	0.58	0.57	-	21.9	5.81	2.69	5(3)
Inner Secretory Cell	0	8.8	3.4	8.8	7.2	15.6	-	13.0	6.9	0.18	2.5	0.17	0.23	-	35.5	5.84	6.86	2(5)
	2	11.6	7.6	7.1	2.7	32.2	-	7.7	6.6	-	3.5	-	-	-	21.1	9.75	7.78	2(3)
	4	7.1	7.7	4.6	7.0	20.6	0.40	13.7	6.8	-	2.9	1.4	0.65	-	27.3	7.46	6.40	3(3)
Stalk Cell	0	8.0	-	0.50	8.8	8.5	-	11.7	2.6	26.3	0.45	0.37	0.13	-	32.6	3.25	1.82	1(3)

[a]Column headings are the standard abbreviations for the cellular organelles, as given in the list of abbreviations.

[b]ER(1) gives the length of endoplasmic reticulum profile present as μm per μm^2 ground-plasm area.

[c]ER(2) gives the area of endoplasmic reticulum profile present as μm^2 per μm^3 total cell volume.

[d]No. gives the number of cells analysed and, in parentheses, the number of randomly selected positions per cell.

Table 3.2: The Structure of Mitochondria in the Outer Secretory Cells at Different Stages of the Secretory Cycle

	Days After Stimulation			
	0	2	4	6
Volume of cell occupied (%)	3.63±0.77	9.00±1.20	7.29±0.27	4.94±0.71
Length of perimeter (μm)	2.94±0.14	2.91±0.21	2.85±0.30	2.79±0.15
Length of cristae (μm)	6.70±0.57	10.20±1.26	9.49±1.98	8.41±1.02
Area within perimeter membrane (μm^2)	1.18±0.09	1.15±0.17	0.94±0.14	1.09±0.09
Area within cristae membrane (μm^2)	0.25±0.02	0.40±0.06	0.36±0.07	0.43±0.05
Length of cristae membrane per unit cross-sectional area ($\mu m\ \mu m^{-2}$)	5.81±0.45	9.02±0.38	9.78±0.61	7.69±0.60
Area of cristae per unit volume ($\mu m^2\ \mu m^{-3}$)	49.18±12.71	68.89±6.66	82.40±12.59	62.80±9.74
Number of mitochondria analysed	12	9	12	11

Table 3.3: Dimensions of Plasmodesmata in Some Glandular Systems

Species	Cellular Boundary	Diameter nm ± s.e. (No.)	Source
Dionaea muscipula	Basal/Basal	52.4±2.4 (8)*	Robins, this study
	Stalk/Basal	84.4±6.1 (9)*	
	Stalk/Inner Secretory	76.1±2.6 (35)*	
	Inner/Inner Secretory	49.7±2.8 (11)*	
	Inner/Outer Secretory	56.6±3.1 (17)*	
	Outer/Outer Secretory	64.6±5.0 (7)*	
Drosera capensis	Stalk/Endodermal	57.1±3.3 (31)	Gilchrist (1974) and unpublished micrographs
	Endodermal/Inner Secretory	51.0±2.0 (3)	
	Endodermal/Inner Secretory	58.8±7.2 (4)	Ragetli et al (1972)
	Stalk/Endodermal	44.7±2.3 (3)	
	Stalk/Stalk	79.0±0.0 (2)	Williams, S.E., and Pickard, B.G. (1974) Planta, 116: 1-16
	Endodermal/Inner Secretory	61.3±6.3 (3)	
	Secretory/Secretory	52.6±2.3 (9)	
Phytollaca americana	-	49.2±8.2 (3)	Dumas, C., Perrin, A., Rougier, M., and Zandonella, P. (1974) Port. Acta biol. ser. A. 14: 501-520
Saponaria officinalis	-	56.0±0.0 (3)	
Tamarix aphylla Tillandsia useneoides	Collecting/Secreting Hair cell walls	80 50 - 70	Robards (1976)
Plumbago capensis	Secretory/Secretory	75 (1)	Rachmilevitz, T., and Joel, D.M. (1976) Israel J. Bot., 25: 127-129
Tropaelum majus	-	71.0±7.4 (4)	Rachmilevitz, T., and Fahn, A. (1975) Ann. Bot., 39: 721-728
Lonicera japonica	-	51.0±6.9 (5)	Fahn, A., and Rachmilevitz, T. (1975) Ann. Bot., 39: 975-976

*Measurements made from negatives. All other measurements taken from prints using stated magnification.

Figure 3.1 Scanning electron micrograph of part of the adaxial surface of the trap showing the semi-sessile digestive glands. Note how there is a fissure between the gland head and the leaf surface (arrows).

Unstimulated tissue: critical point dried and gold coated. x 225.

Figure 3.2 Scanning electron micrograph of two digestive glands.

Unstimulated tissue: critical point dried and gold coated. x 625.

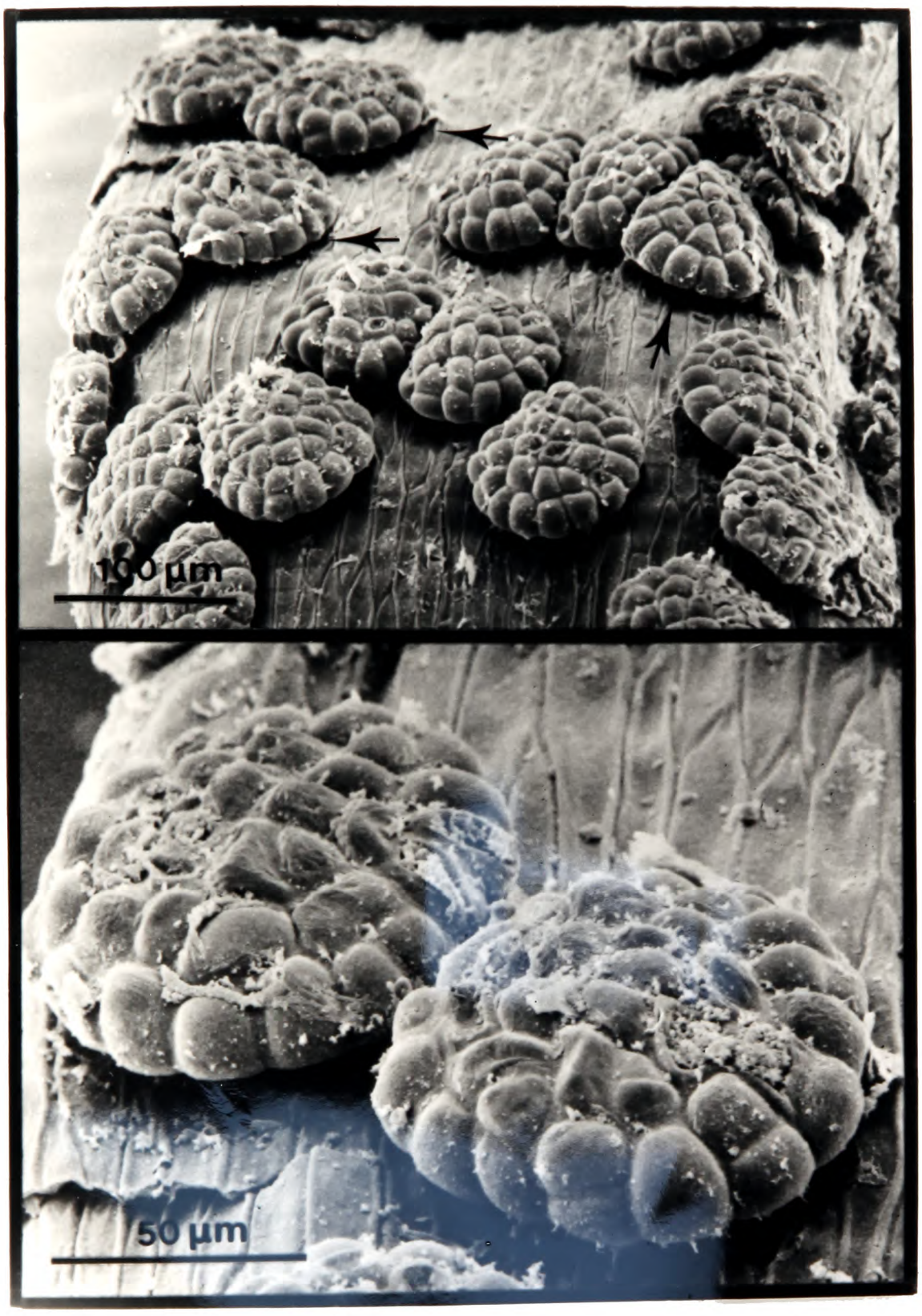

100 µm
50 µm

Figure 3.3 Scanning electron micrograph of a digestive gland showing the surface structure.

Unstimulated tissue: critical point dried and gold coated. x 1,025.

Figure 3.4 Scanning electron micrograph of a digestive gland showing the detail of the cellular surface. Note the irregular allantoid ridging over the whole surface of the cell. The light, nodular material in the lower part of the micrograph is fungal mycelia.

Unstimulated tissue: critical point dried and gold coated. x 7,470.

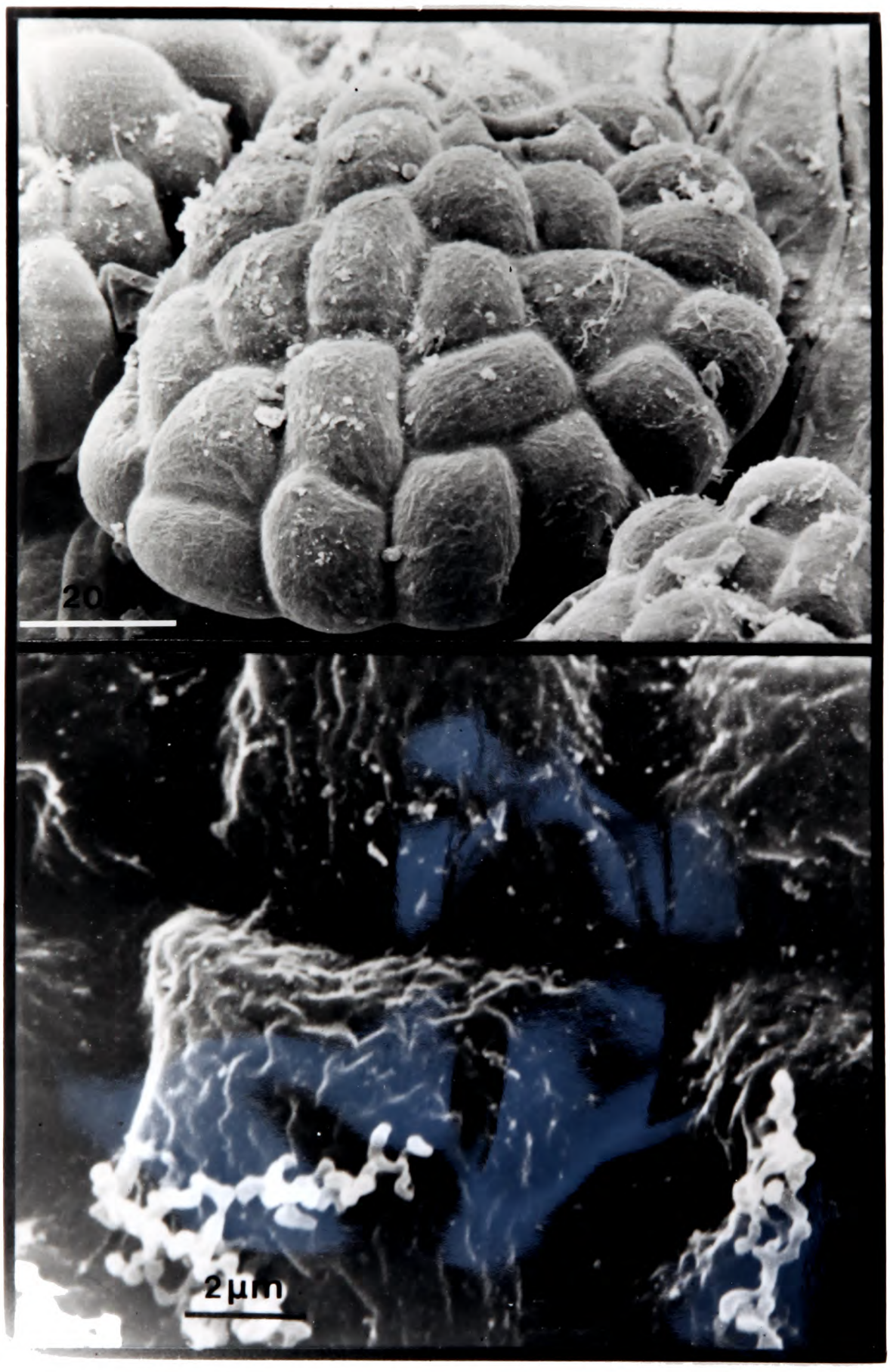

20
2µm

Figure 3.5 Transverse section through a digestive gland showing the basic features of the ultrastructure. The gland head, composed of 2 layers of secretory cells (OSC : ISC) is supported on a stalk of 4 auxiliary cells, 2 stalk cells (SC) and 2 basal cells (BC). The latter are embedded in the epidermis (E) and have symplastic connections to both the epidermal and sub-epidermal (SE) cells. The endocuticle is marked with stars.

Unstimulated tissue: Epikote-812 resin: uranyl acetate - lead citrate poststain. x 2,640.

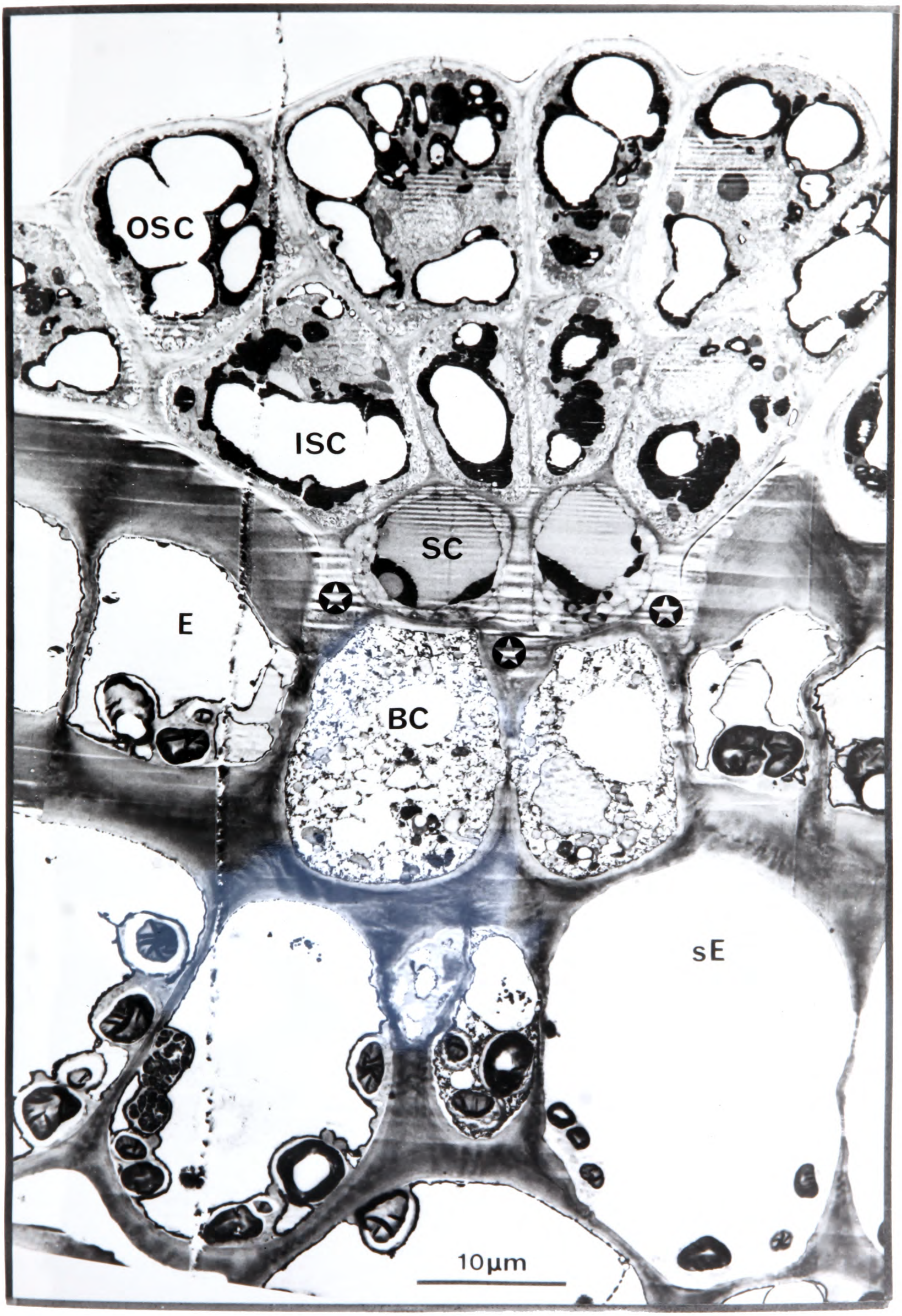

OSC
ISC
SC
E
BC
sE
10µm

Figure 3.6 Transverse section through a basal cell showing the general features of its structure. Pits containing plasmodesmata are found connecting the 2 basal cells with each other and all surrounding cells (arrows).

Unstimulated tissue: Epikote-812 resin: lead citrate poststain. x 3,710.

Figure 3.7 Transverse section of an epidermal cell (lower part) adjacent to a basal cell (upper part) showing the structure of the chloroplasts in the epidermal cells. For a comparison with the plastids in the gland cells see Figs. 3.19 and 3.25 to 3.27.

Stimulated tissue - day 2: Spurr's resin: uranyl acetate - lead citrate poststain. x 26,400.

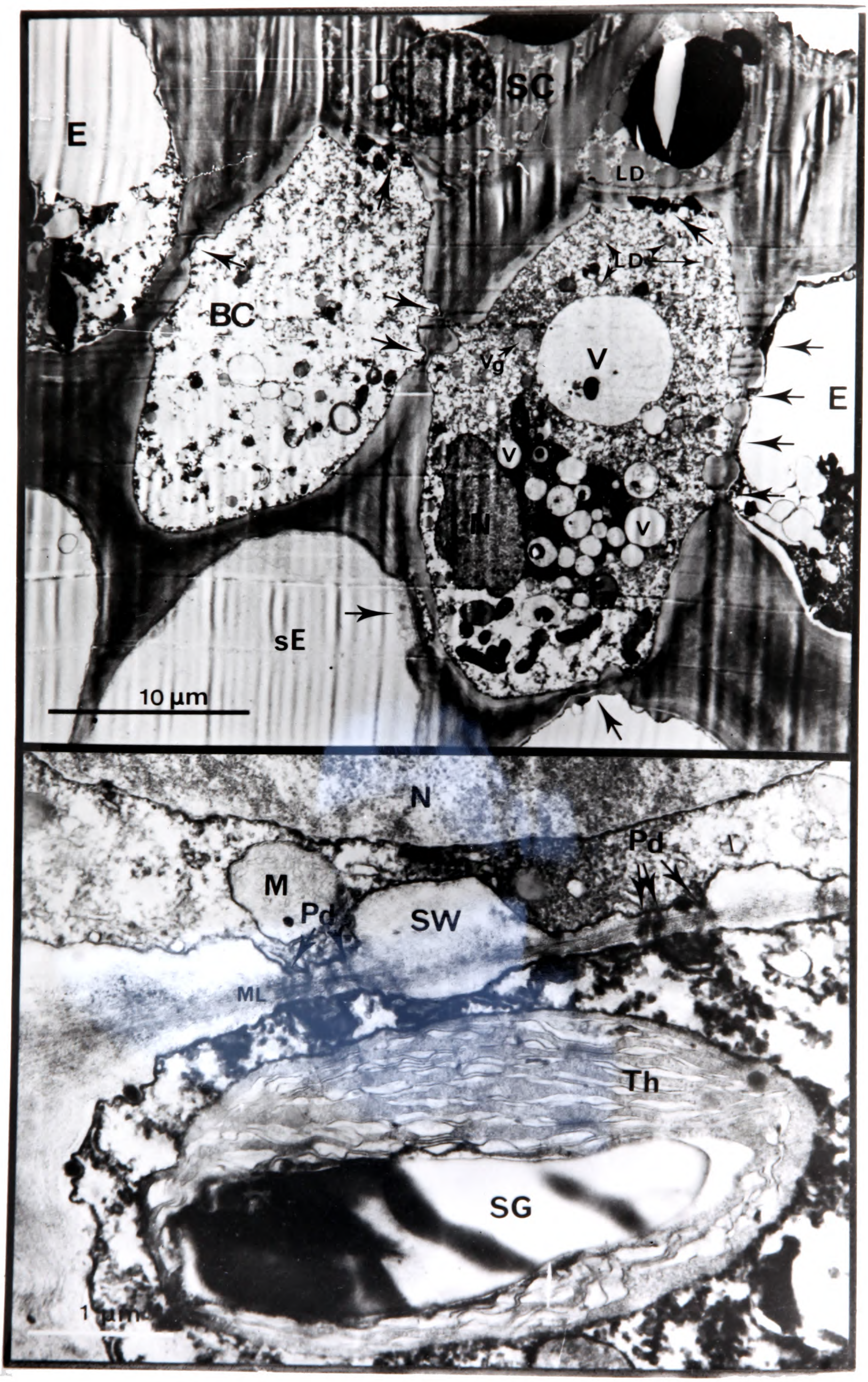

SC
E
LD
LD
BC
Vg
V
E
V
V
N
sE
10 µm
N
Pd
M
Pd
SW
ML
Th
SG
1 µm

Figure 3.8 Detail from a basal cell showing part of a pit field. Note the dictyosome, seen in glancing section, and vesicles from it arriving at the cell periphery and apparently fusing with the plasmalemma (arrows). Note also the numerous plasmodesmata.

Unstimulated tissue: Spurr's resin: uranyl acetate - lead citrate poststain. x 44,590.

Figure 3.9 Detail from a basal cell showing numerous vesicles, probably derived from the dictyosomes, arriving at the cell periphery and apparently fusing with the plasmalemma (arrows).

Unstimulated tissue: Spurr's resin: uranyl acetate - lead citrate poststain. x 55,870.

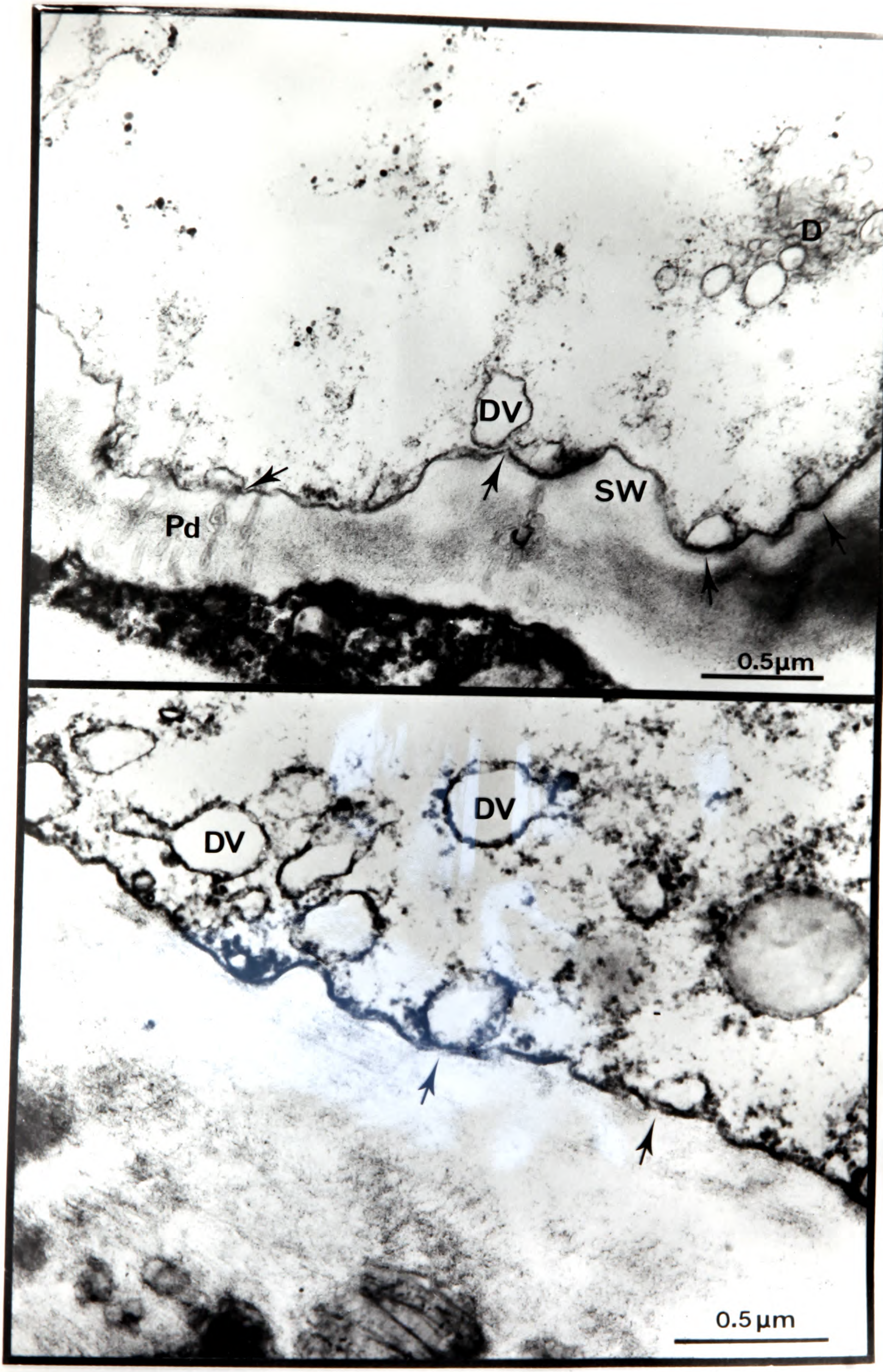

D
DV
SW
Pd
0.5µm
DV
DV
0.5 µm

Figure 3.10 Transverse median section showing part of a stalk cell and small parts of the adjacent basal cell and inner secretory cell. The pits between the stalk cell and the basal cell are marked with arrows. Note the even size and texture of the lipid droplets.

Unstimulated tissue: Spurr's resin: uranyl acetate - lead citrate poststain. x 11,880.

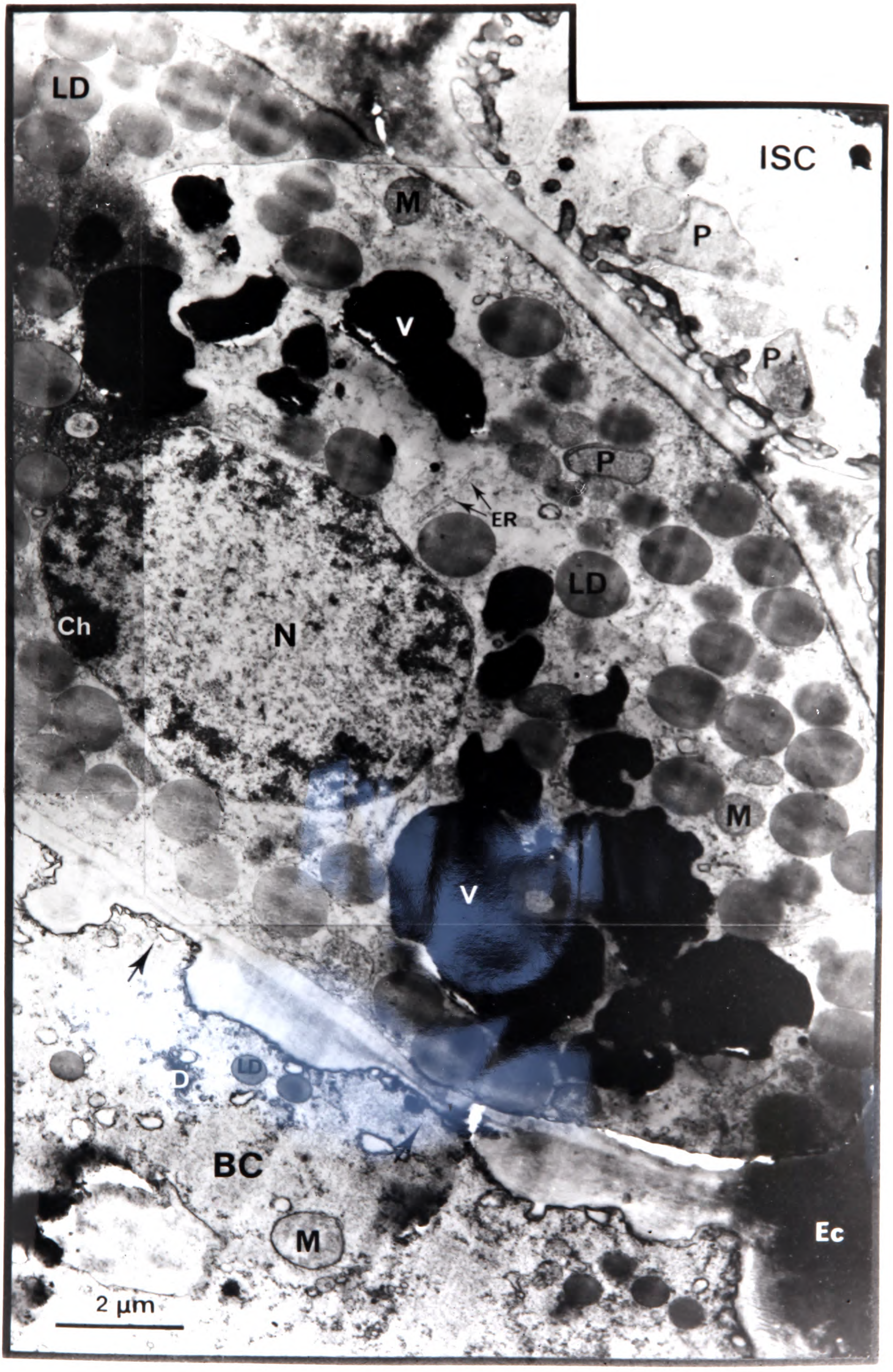

LD
ISC
M
P
V
P
P
ER
LD
Ch
N
M
V
D
LD
BC
M
Ec
2 µm

Figure 3.11 Detail of the cytoplasm of a stalk cell showing the lipid droplets. Note the totally amorphous texture and the single boundary line (arrows).

Unstimulated tissue: Epikote-812 resin: lead citrate poststain. x 36,420.

Figure 3.12 Detail of the cytoplasm of a stalk cell from the same preparation as Fig. 3.11 following treatment of the thin section with lipase solution for 90 min. Note that the boundary of each droplet now has a tri-layered appearance (arrows), and that the contents have been largely destroyed. The dark globules are probably caused by the products of the lipolysis adhering to the section.

Unstimulated tissue: Epikote-812 resin: uranyl acetate - lead citrate poststain. x 36,740.

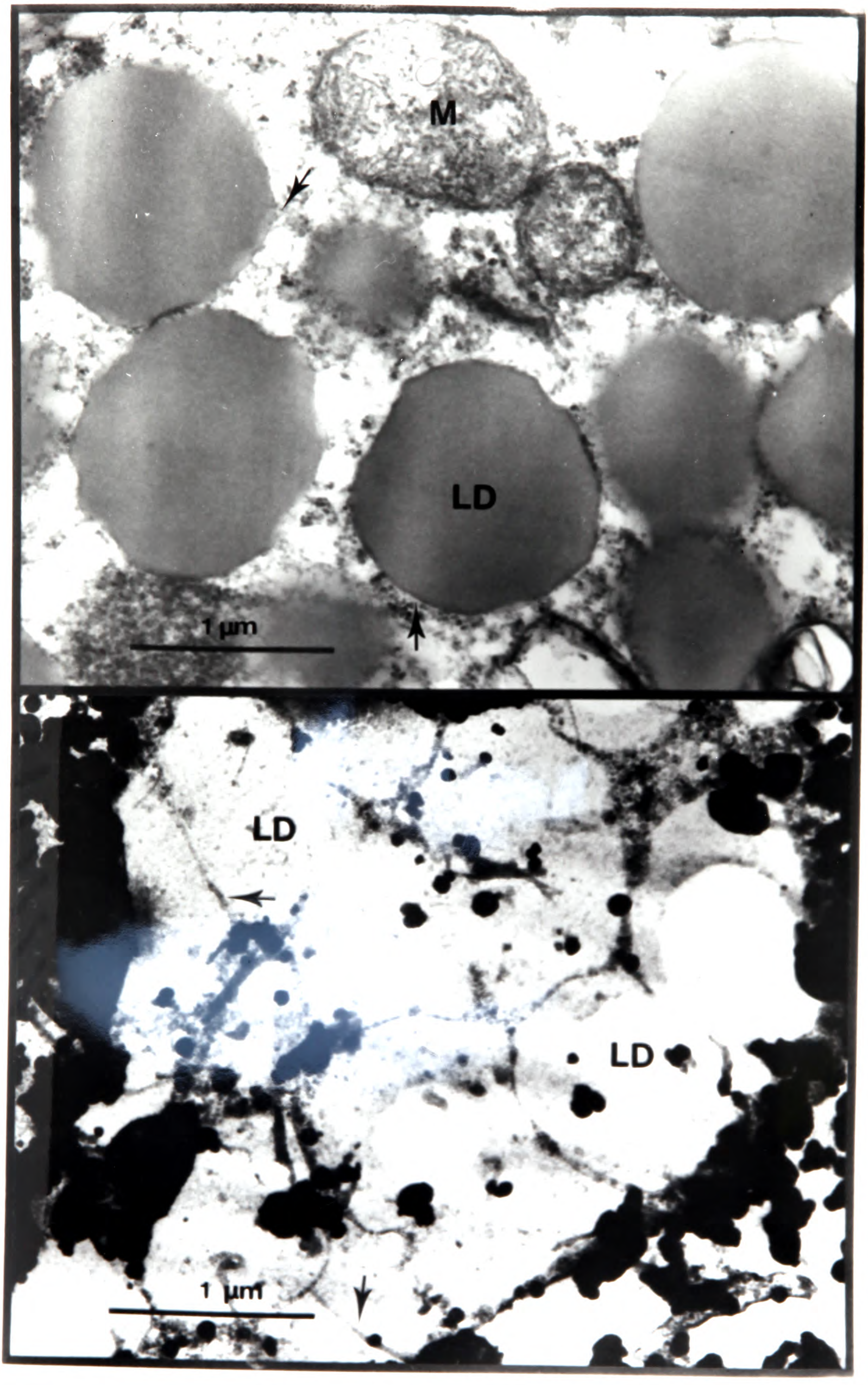

M
LD
1 µm
LD
LD
1 µm

Figure 3.13 A low magnification view of a stalk cell after 90 minutes treatment of the thin section with lipase solution. Note how the dark globules are concentrated over the vacuole and endocuticles.

Unstimulated tissue: Epikote-812 resin: uranyl acetate - lead citrate poststain. x 7,250.

Figure 3.14 A section from the same block as Fig. 3.13 treated for 15 min with hydrogen peroxide only. This has had negligible effect on the lipid droplets.

Unstimulated tissue: Epikote-812 resin: uranyl acetate - lead citrate poststain. x 11,600.

Figure 3.15 A control section from the same block as Figs. 3.13 and 3.14 but untreated.

Unstimulated tissue: Epikote-812 resin: uranyl acetate - lead citrate poststain. x 8,430.

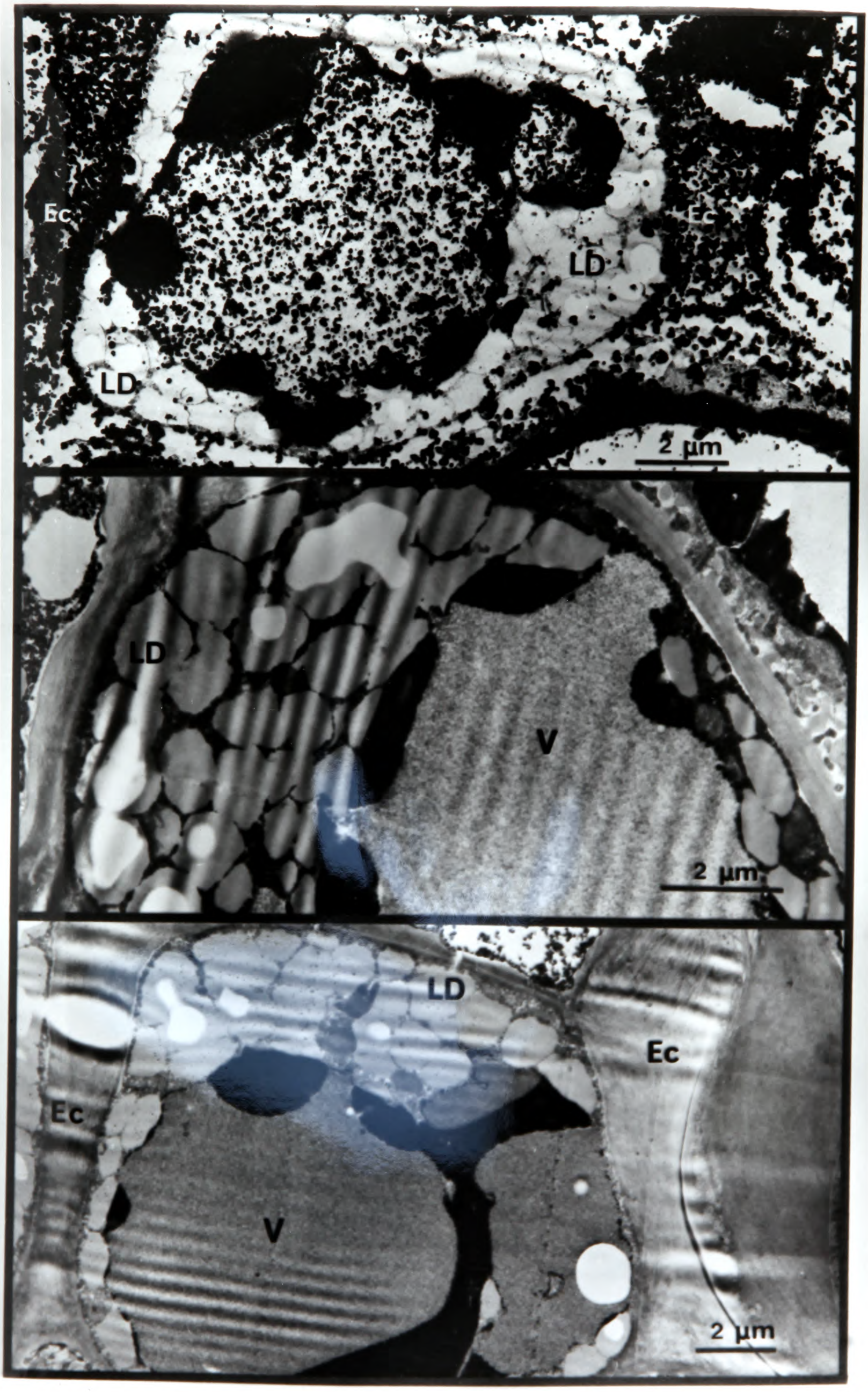

Ec
LD
Ec
LD
2 µm
LD
V
2 µm
LD
Ec
Ec
V
2 µm

Figure 3.16 Transverse section through the lower part of a gland showing the endocuticle surrounding the stalk cells. Note how the wall to either side of the stalk cells is completely permeated by this dense amorphous deposit. Note also how the endocuticle is continuous with the cuticle of both the secretory cells and the epidermis.

Unstimulated tissue: Epikote-812 resin: lead citrate poststain. x 5,900.

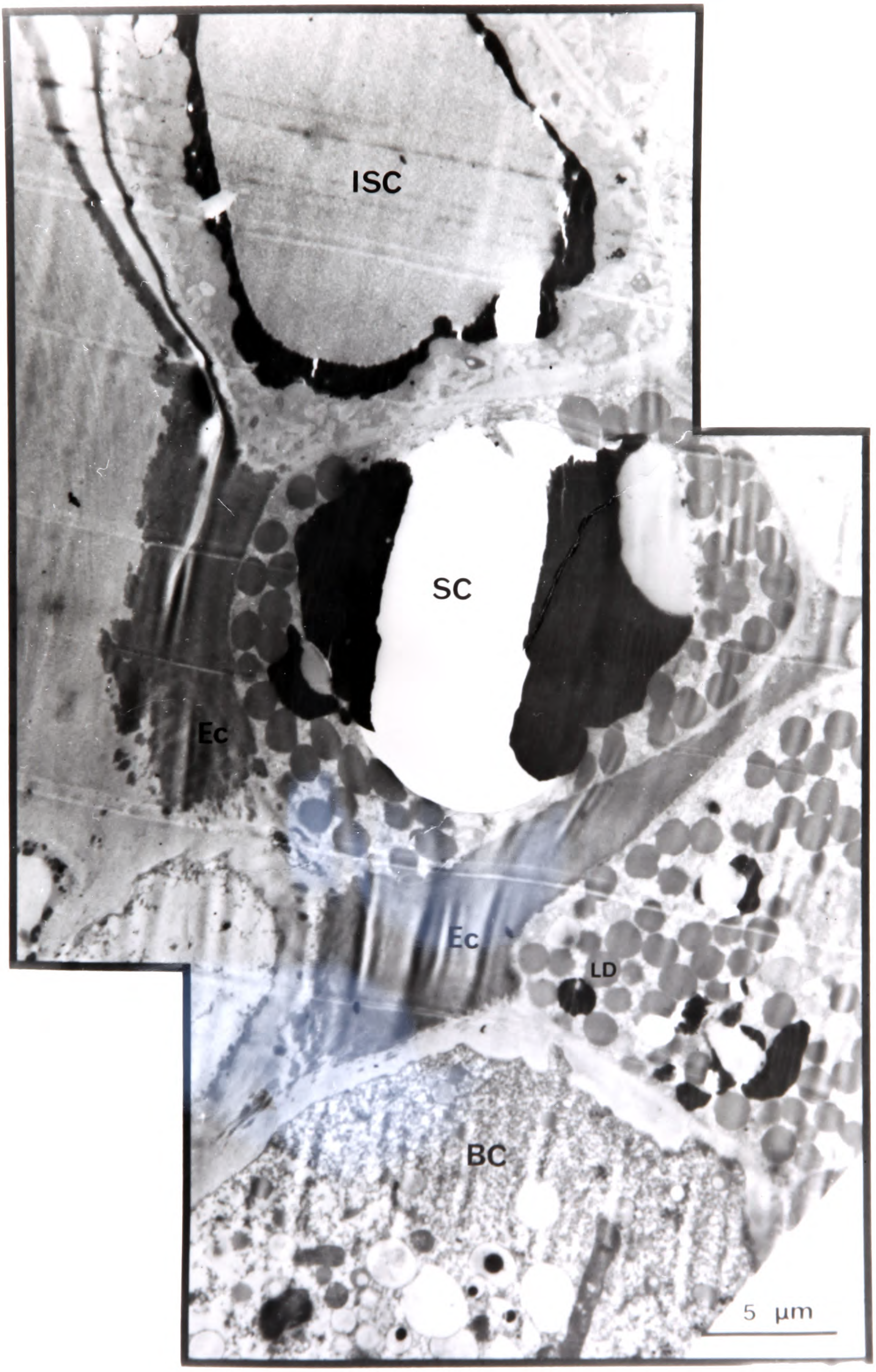
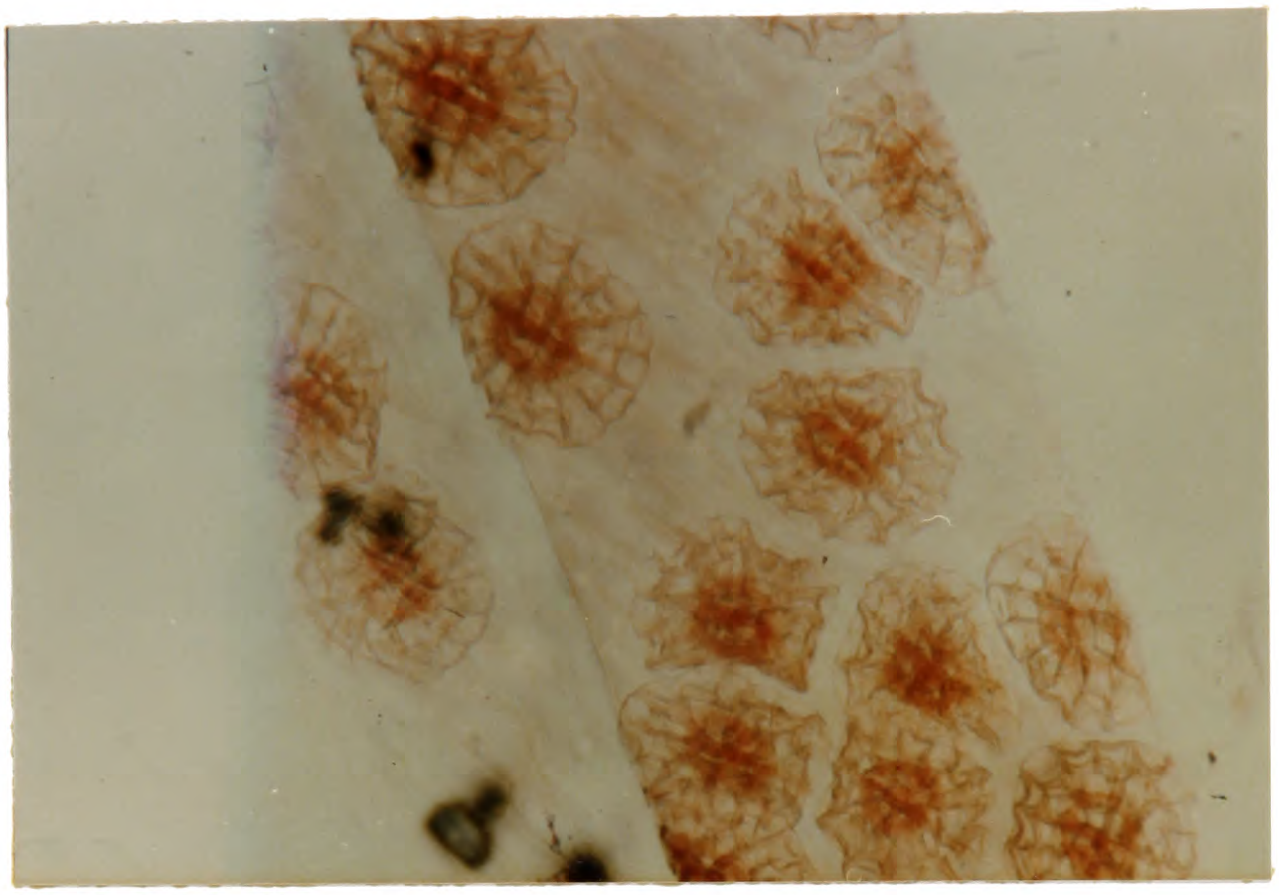

ISC
SC
Ec
Ec
LD
BC
5 µm

Figure 3.17 Light micrograph showing the reaction of fresh tissue with Sudan IV. Note the heavy staining of the walls at the centre of the gland.

Fresh tissue. x 100.

Figure 3.18 Light micrograph showing the distribution of suberin in the gland by the method of Johansen (1940).

Fresh tissue. x 120.



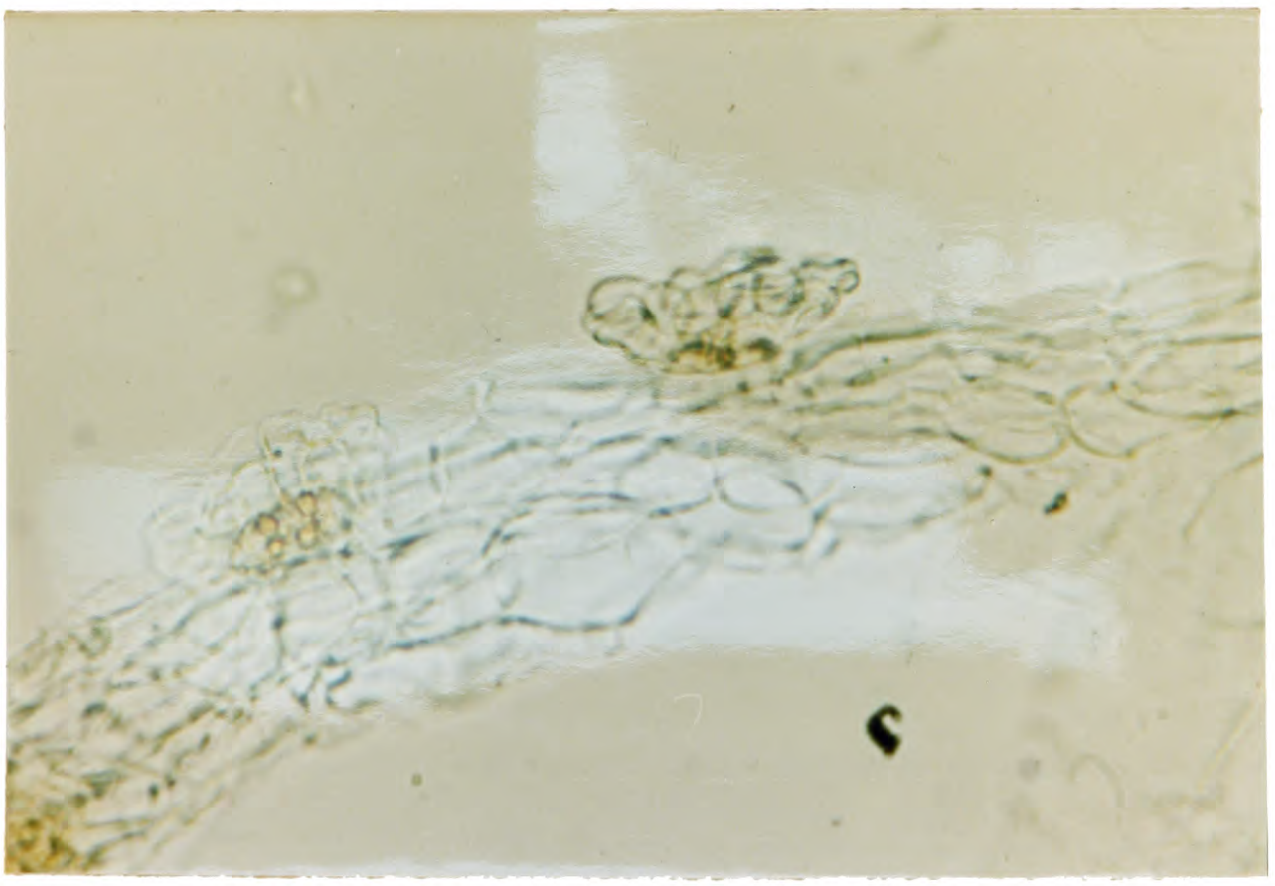


Figure 3.19 General transverse section through an outer and parts of two inner secretory cells, showing the general features of these cells.

Stimulated tissue - day 4: Spurr's resin: lead citrate poststain. x 9,250.

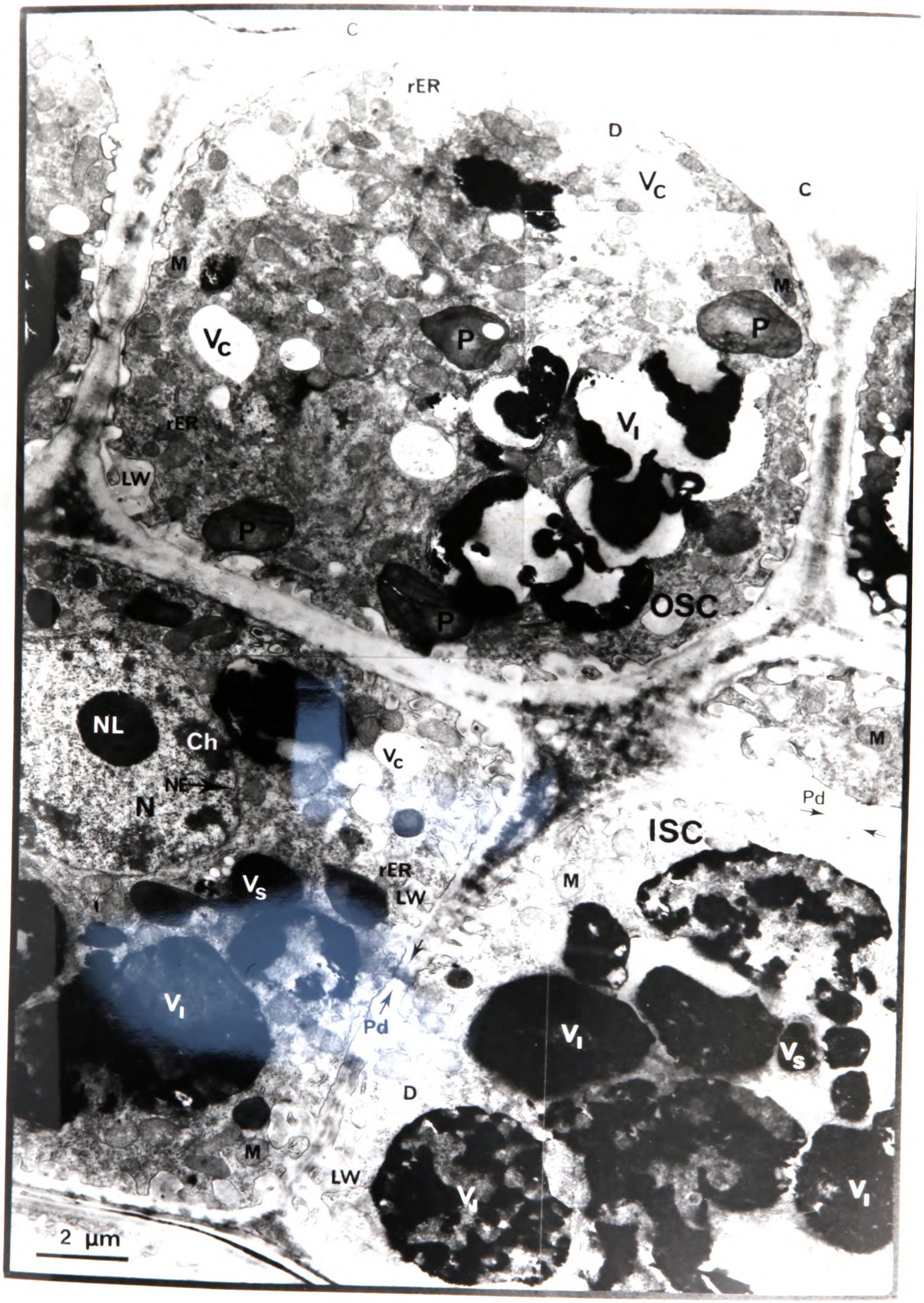

C
rER
D
V_C
C
M
M
V_C
P
P
rER
V_I
LW
P
P
OSC
NL
Ch
NE
N
V_C
M
Pd
ISC
rER
LW
V_S
M
V_I
Pd
V_I
V_S
D
M
LW
V_I
V_I
2 µm

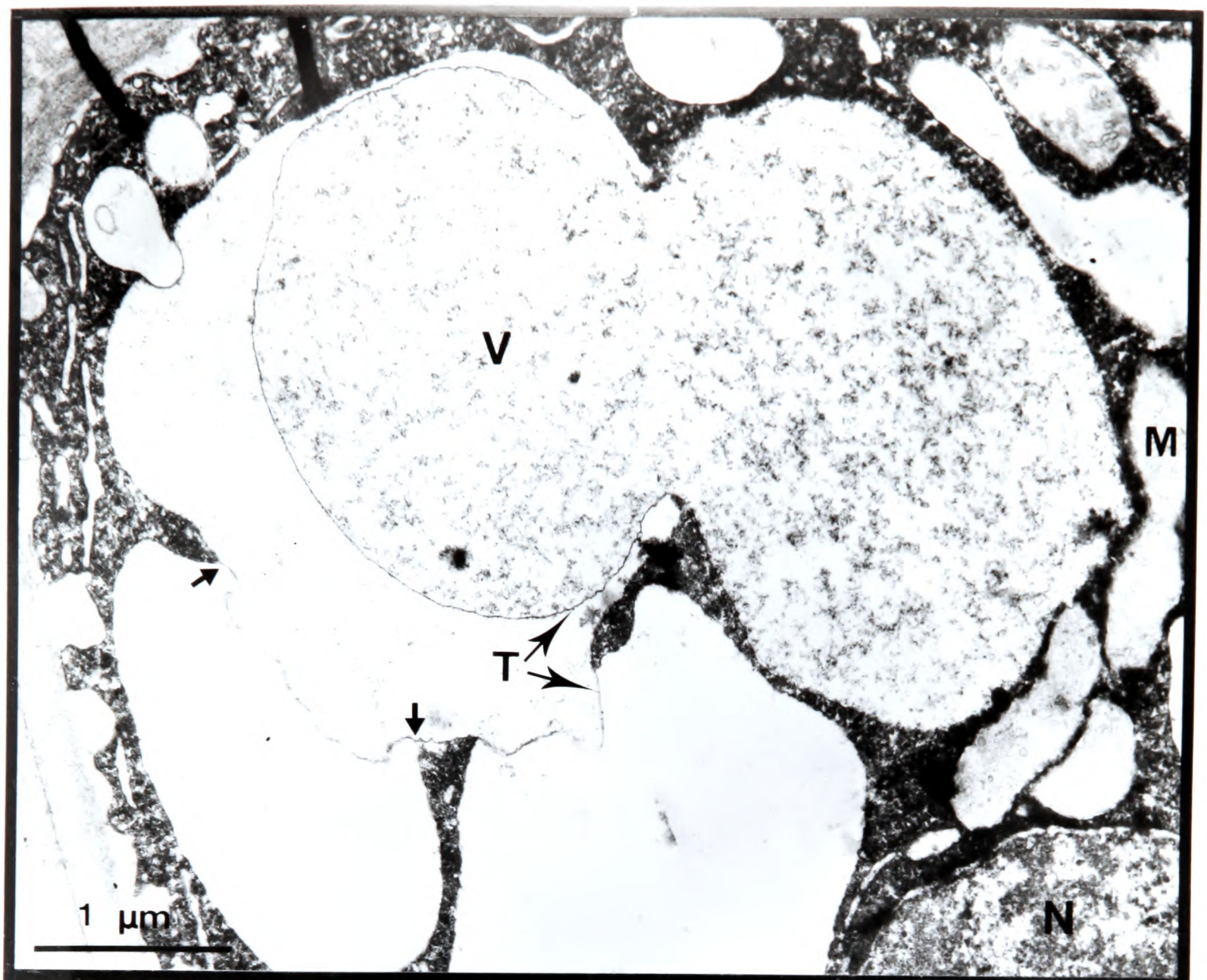
Figure 3.20 Detail from the cytoplasm of an inner secretory cell showing several different species of vacuole in close juxtaposition. Note the clear, single tonoplast separating each vacuolar compartment. Between the short arrows (→) the two tonoplasts are tightly apposed without fusing.

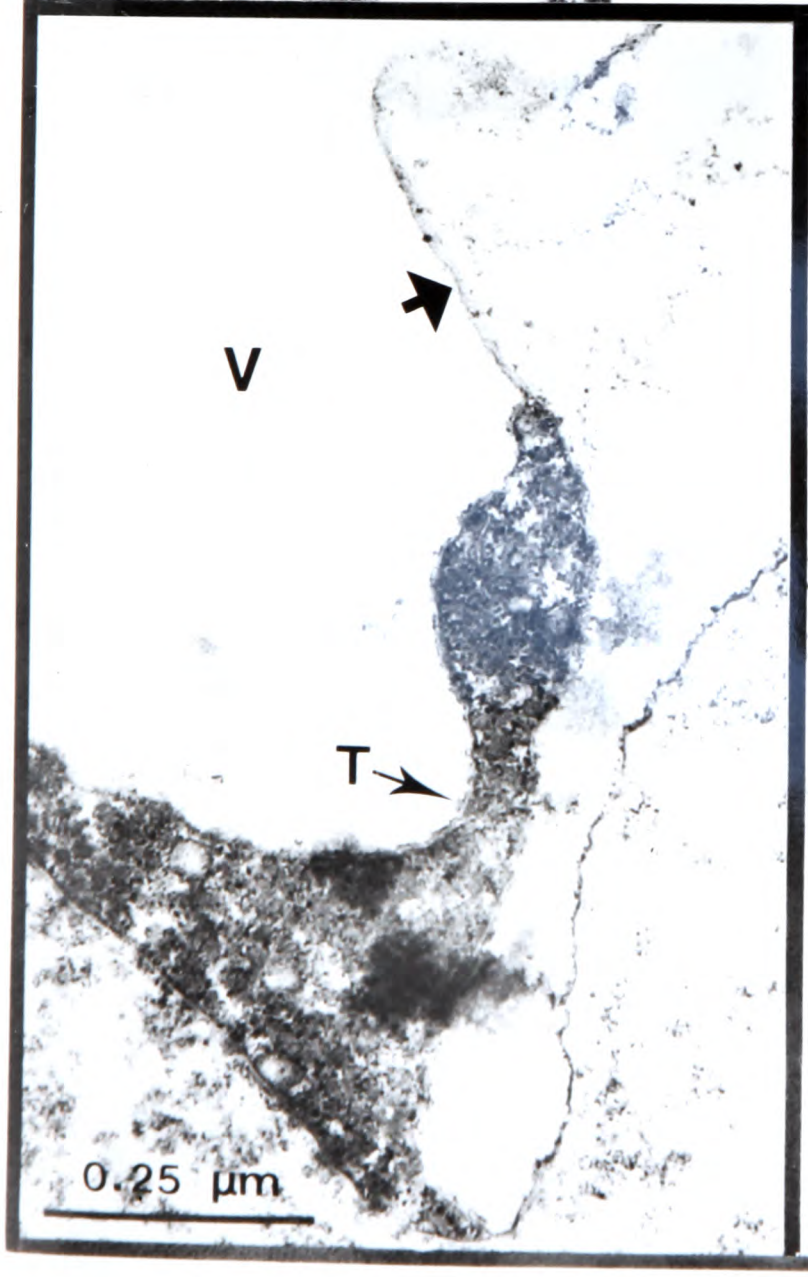
Unstimulated tissue: Spurr's resin: lead citrate poststain. x 26,110.

Figure 3.21 Detail from Fig. 3.20 showing the tonoplasts of the 3 compartments. The double tonoplast is marked with a heavy arrow (⇒).
x 107,200.

Figure 3.22 Detail from the cytoplasm of an inner secretory cell showing a membranous body formed against the tonoplasts at the junction of two vacuolar compartments.

Unstimulated tissue: Spurr's resin: lead citrate poststain. x 197,000.


V
M
T
N
1 µm


V
T
0.25 µm

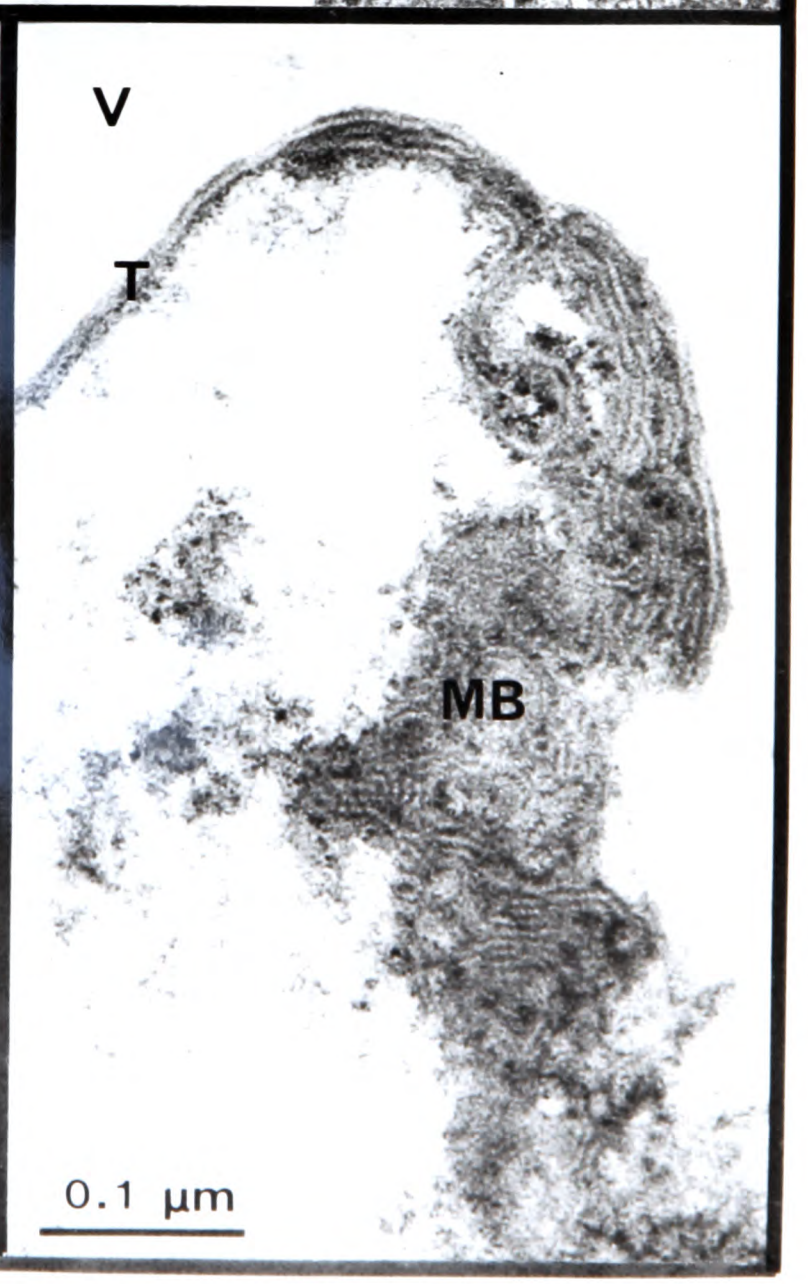

V
T
MB
0.1 µm

Figure 3.23 General transverse section of part of the cytoplasm of an outer gland cell.

Unstimulated tissue: Spurr's resin: uranyl acetate - lead citrate poststain. x 29,090.

Figure 3.24 Oblique section through the edge of the nuclear envelope showing the nuclear pores in longitudinal section. Note how the underlying membrane, although not resolved, gives a darker stain compared to the surrounding cytoplasm.

Stimulated tissue - day 6: Spurr's resin: uranyl acetate - lead citrate. x 47,410.

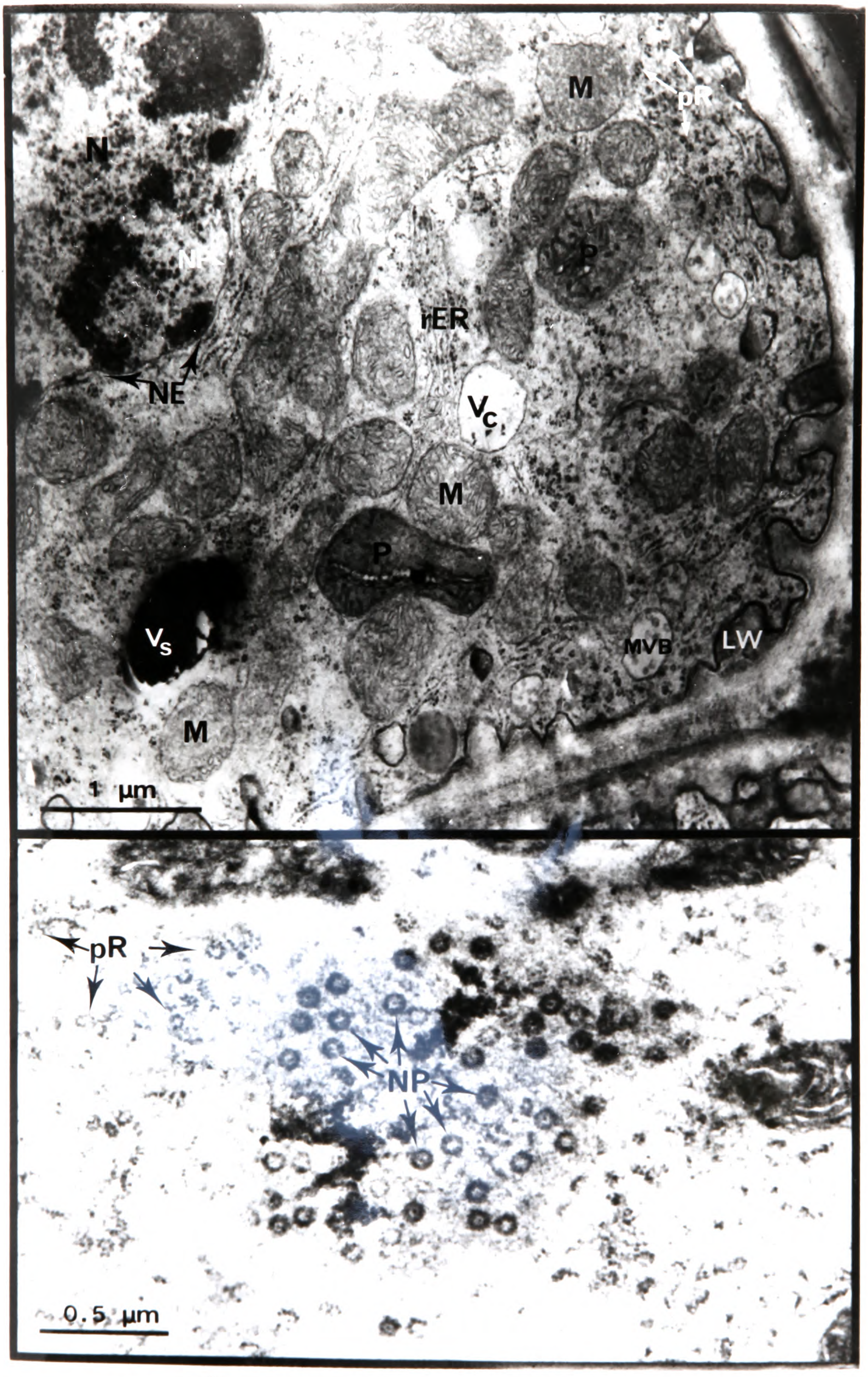

M
pR
N
NP
P
rER
NE
V_C
M
P
V_S
MVB
LW
M
1 µm
pR
NP
0.5 µm

Figure 3.25 Plastid in an outer secretory cell. White arrows mark the tubules noted by Scala et al (1968a; 1968b).

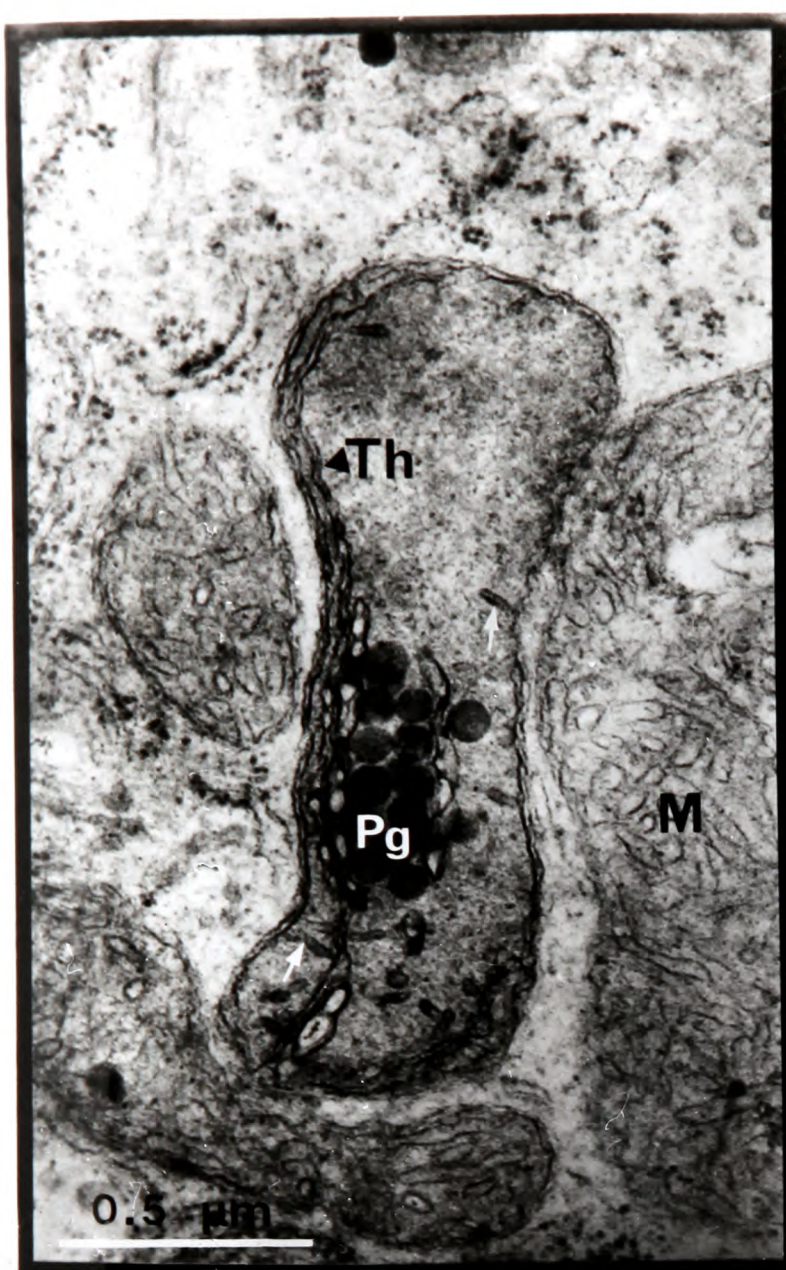
Unstimulated tissue: Spurr's resin: lead citrate post-stain. x 50,400.

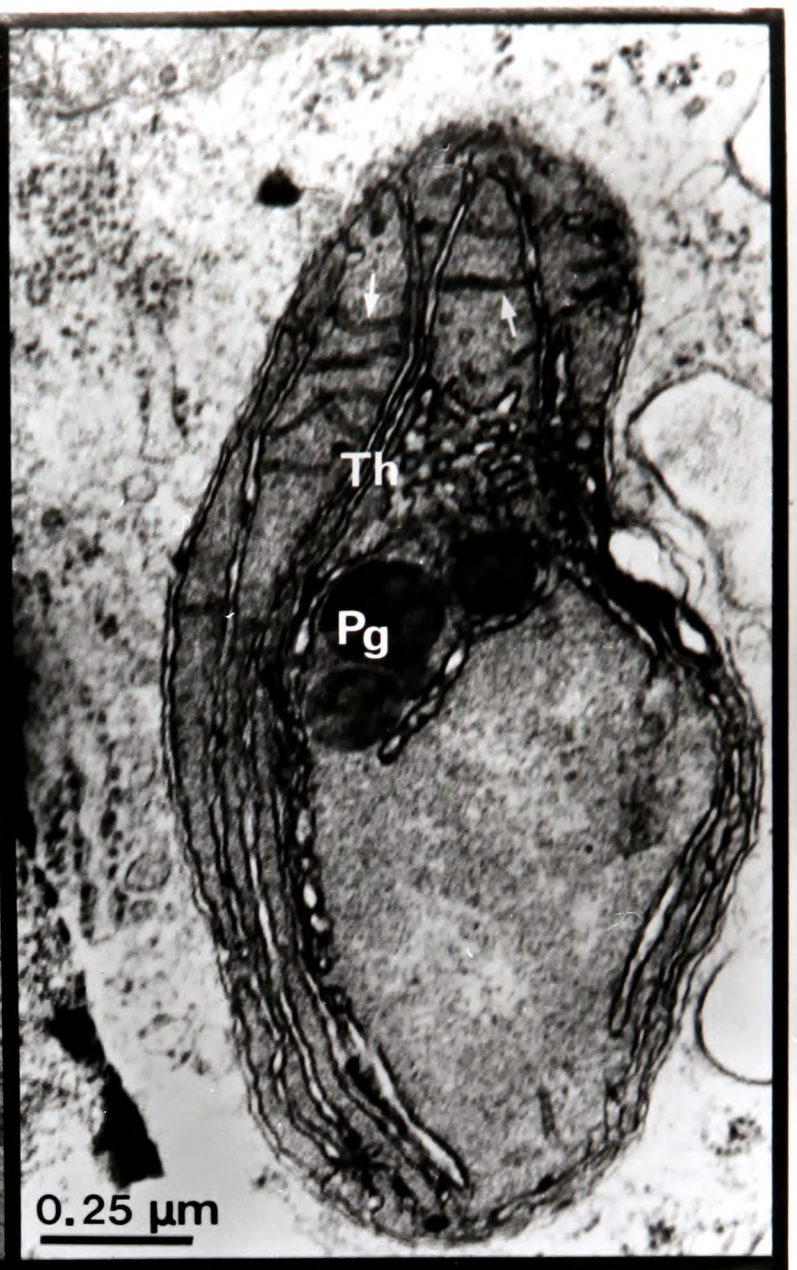
Figure 3.26 Plastid in an inner secretory cell. White arrows mark the tubules noted by Scala et al (1968a; 1968b).

Stimulated tissue - day 4: Spurr's resin: lead citrate poststain. x 58,560.

Figure 3.27 Plastid in an inner secretory cell showing a highly developed tubular complex. White arrow marks a tubule as noted by Scala et al (1968a; 1968b).

Unstimulated tissue: Spurr's resin: uranyl acetate - lead citrate poststain. x 127,600.


Th
Pg
M
0.5 µm


Th
Pg
0.25 µm

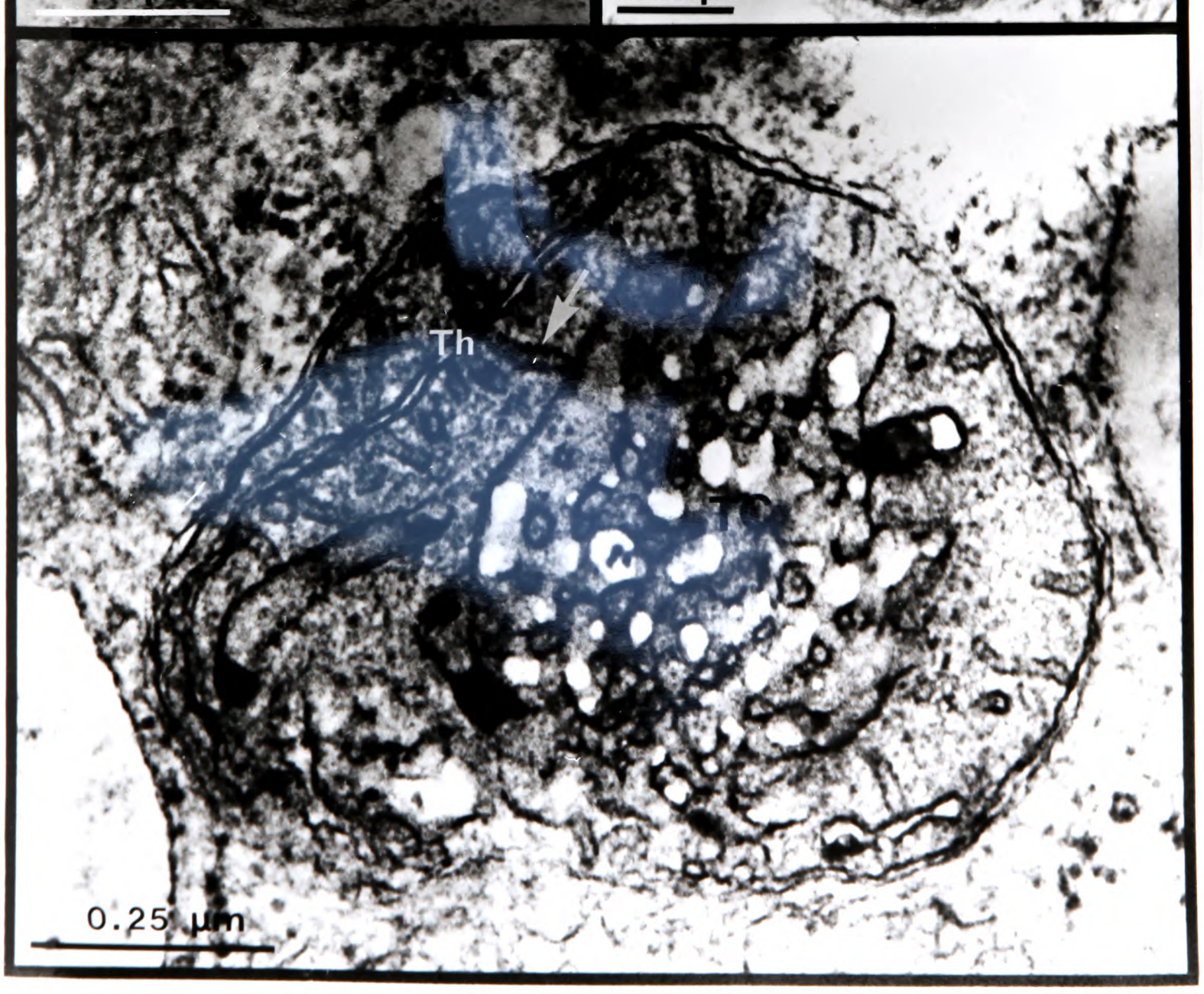

Th
0.25 µm

Figure 3.28 Stacked rough endoplasmic reticulum lying along the cell wall of an inner secretory cell on the side facing an outer secretory cell. Note the point of fusion to the plasmalemma (arrow) and the irregular width of the cisternae.

Unstimulated tissue: Spurr's resin: uranyl acetate - lead citrate poststain. x 79,470.

Figure 3.29 Stacked rough endoplasmic reticulum lying along the cell wall between 2 outer secretory cells. Note the points of fusion to the plasmalemma (arrows) and the irregular width of the cisternae. In the upper cell is a dictyosome apparently undergoing division.

Unstimulated tissue: Spurr's resin: uranyl acetate - lead citrate poststain. x 45,530.

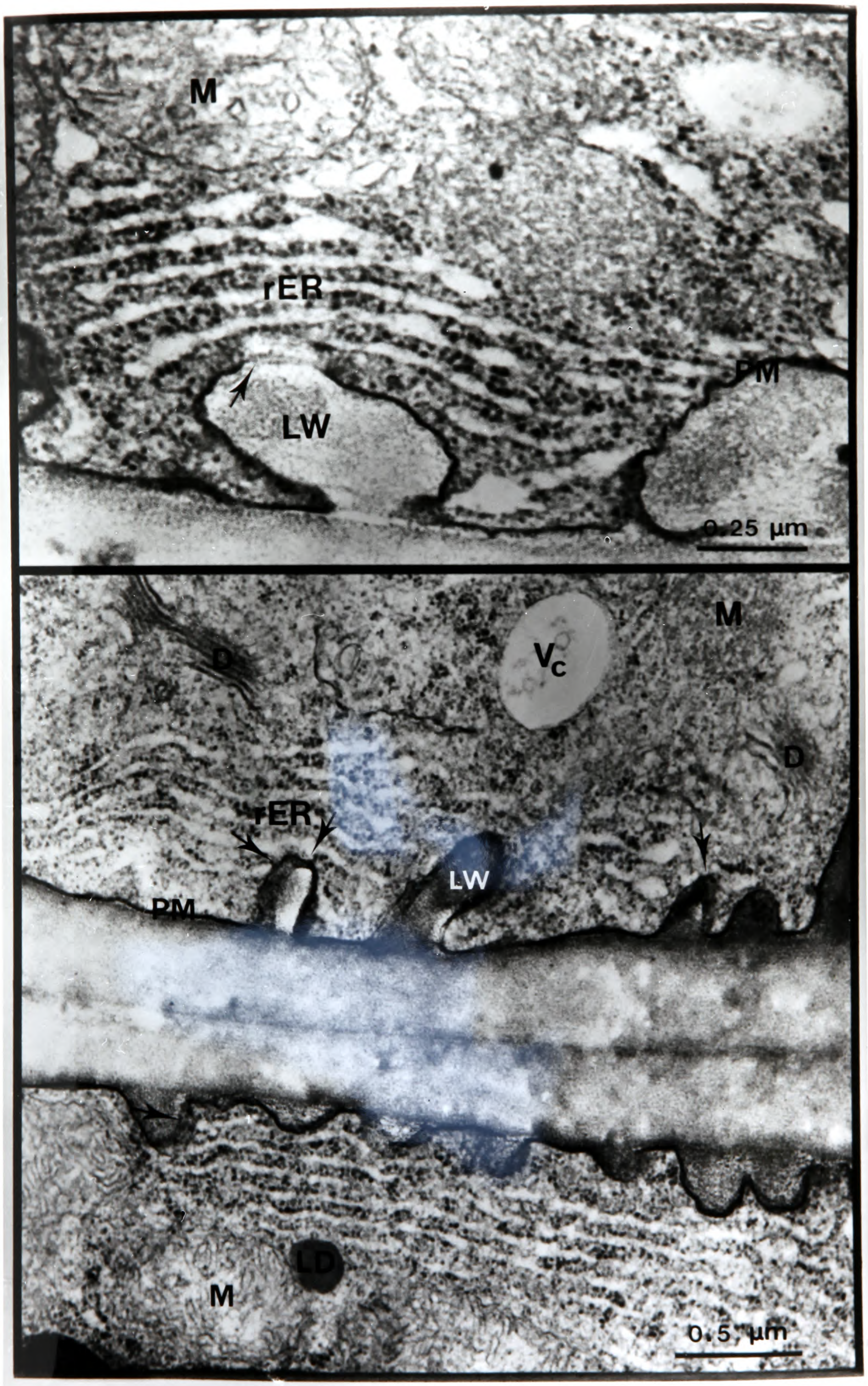

M
rER
LW
PM
0.25 µm
M
D
V_C
rER
LW
PM
LD
M
0.5 µm

Figure 3.30 Longitudinal section through the cell wall between two inner secretory cells showing rough endoplasmic reticulum in close association with a plasmodesmata. Arrow marks a point of fusion to the plasmalemma.

Unstimulated tissue: Epikote-812 resin: lead citrate poststain. x 53,300.

Figure 3.31 Stacked rough endoplasmic reticulum in the corner of an inner secretory cell showing very even cisternae and numerous points of fusion to the plasmalemma (arrows).

Unstimulated tissue: Spurr's resin: lead citrate poststain. x 38,210.

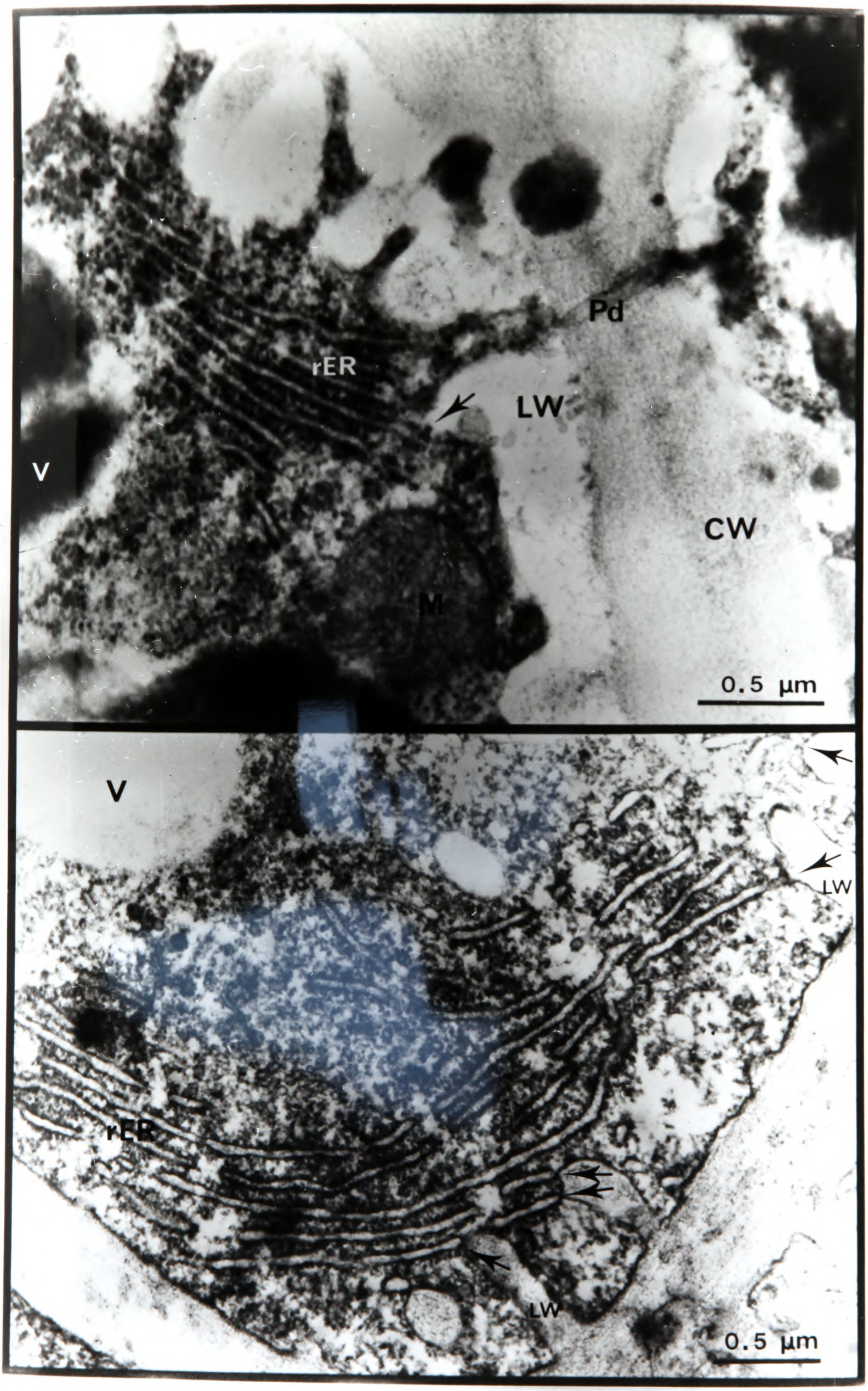

rER
Pd
LW
V
CW
M
0.5 µm
V
LW
rER
LW
0.5 µm

Figure 3.32 Dictyosome in an outer secretory cell in a virtually totally inactive state. The cisternae are of uniform length and width and regularly spaced. Note the inter-cisternal elements (arrows).

Unstimulated tissue: Spurr's resin: lead citrate poststain. x 161,200.

Figure 3.33 Two dictyosomes in an outer secretory cell from the same preparation as Figs. 3.32 and 3.34. The short, closely apposed cisternae end in spherical vesicles which are being lost to the cytoplasm.

Unstimulated tissue: Spurr's resin: lead citrate poststain. x 53,060.

Figure 3.34 A dictyosome from the same outer secretory cell as Fig. 3.33 showing cisternae which are closely apposed and distended along their entire length.

Unstimulated tissue: Spurr's resin: lead citrate poststain. x 118,800.

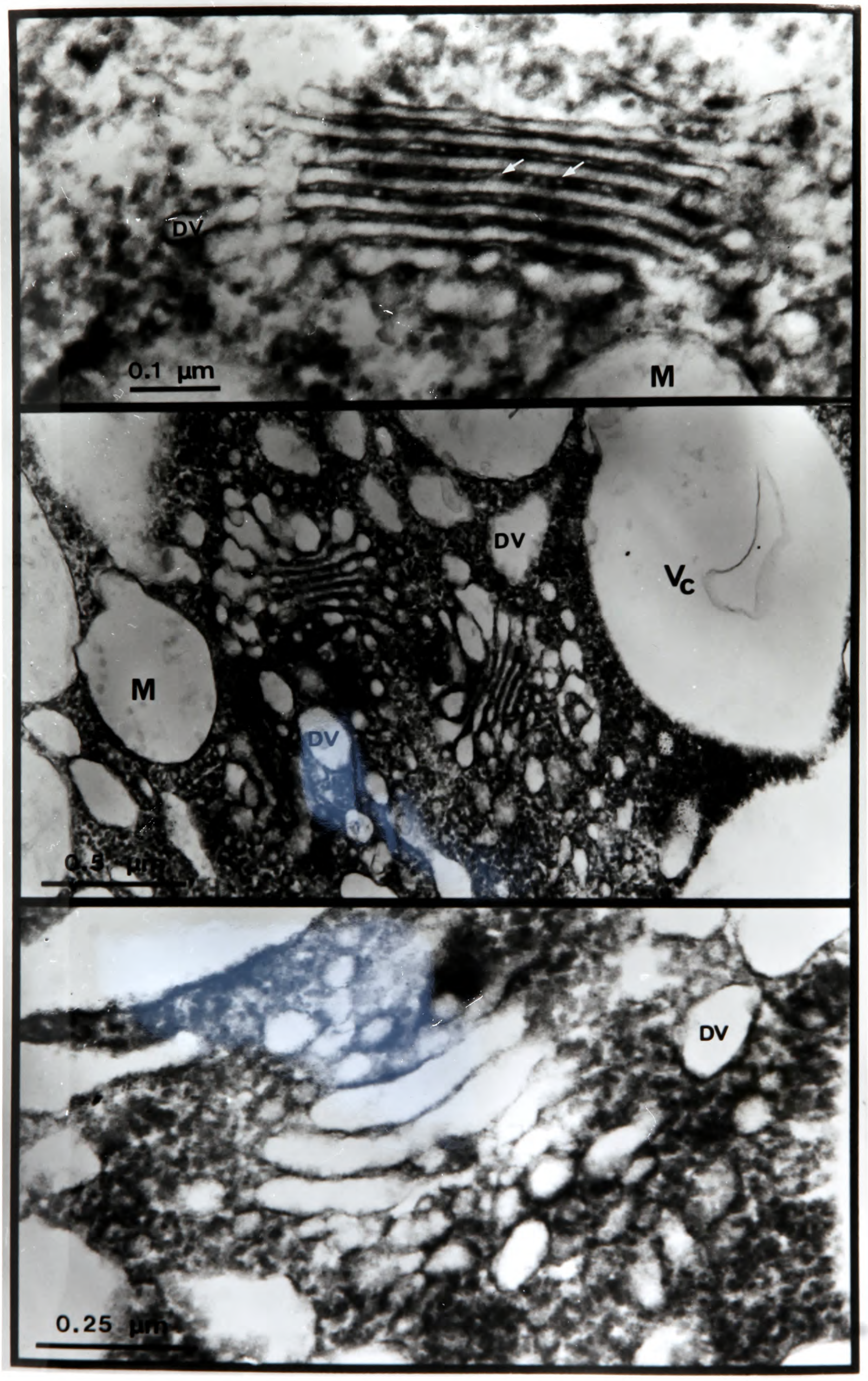

DV
0.1 µm
M
DV
V_C
M
DV
0.5 µm
DV
0.25 µm

Figure 3.35 Extensive labyrinthine wall in the corner of an inner secretory cell (stalk cell to lower centre: outer secretory cells to upper left and upper right). This preparation is unusual in that the labyrinthine wall does not show the characteristic structure (Fig. 3.36) found in most preparations.

Stimulated tissue - day 4: Spurr's resin: uranyl acetate - lead citrate poststain. x 11,430.

Figure 3.36 Detail of the labyrinthine wall in an inner secretory cell showing the basic structure as described in the text. The core material is marked with stars.

Unstimulated tissue: Epikote-812: lead citrate poststain. x 43,180.

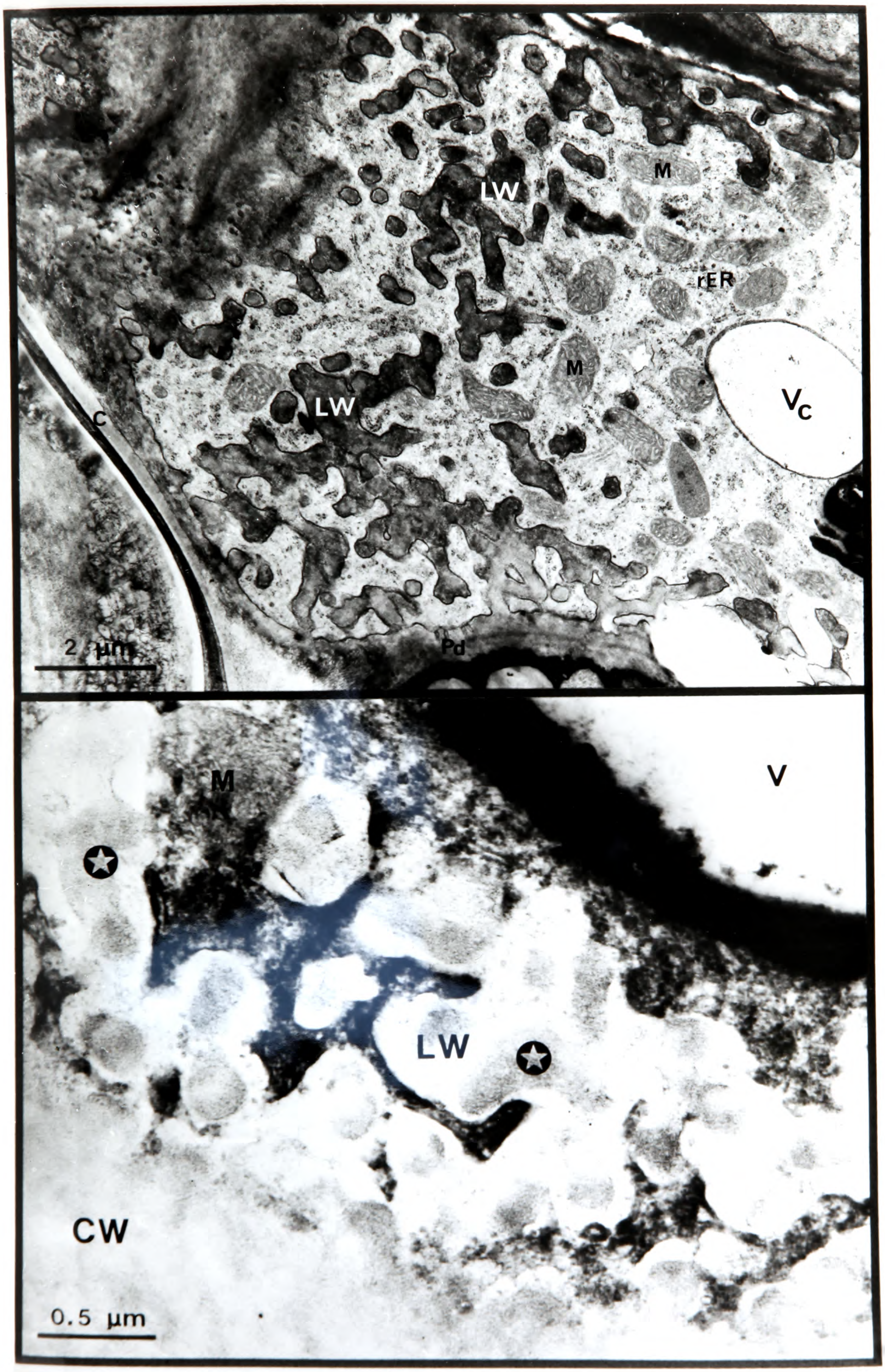

LW
M
rER
M
LW
C
V_C
2 µm
Pd
M
V
LW
CW
0.5 µm

Figure 3.37 A detailed analysis of the labyrinthine walls of the outer and inner layers of secretory cells to examine the correlation between the different features which contribute to their overall architecture.

In each set of paired data the line shows the best-fit linear regression analysis when there is a significant one-tailed correlation coefficient (r) displayed by the data.

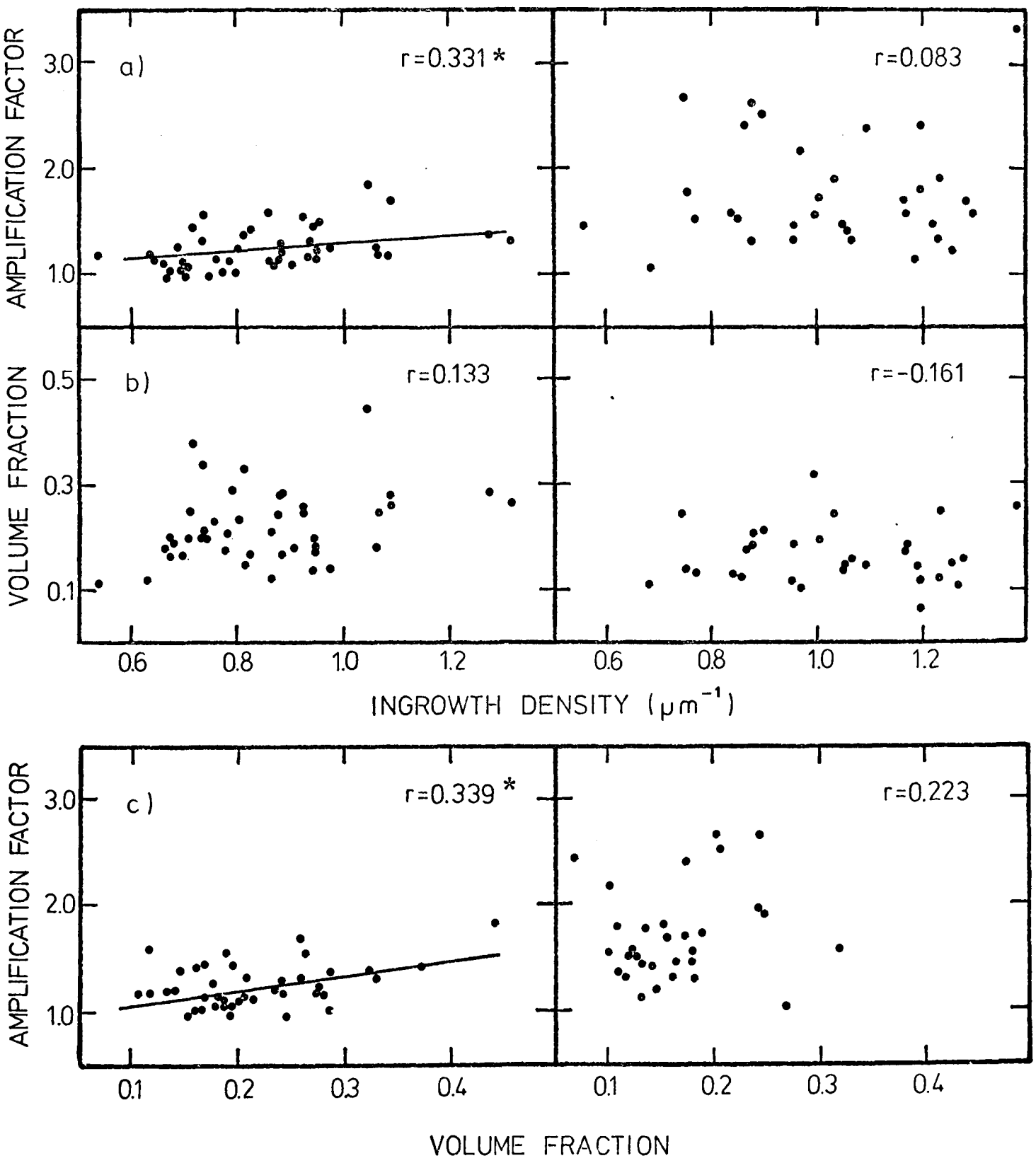
Levels of significance shown are:

* $p<0.05$

** $p<0.01$

*** $p<0.001$

SECRETORY CELL



SECRETORY CELL

OUTER

INNER

d)

r=-0.089

r=-0.168

e)

r=-0.349 *

r=0.086

f)

r=-0.597 ***

r=-0.569 ***

g)

r=0.454 **

r=0.492 **

AMPLIFICATION FACTOR

VOLUME FRACTION

INGROWTH DENSITY (μm^{-1})

TOTAL INGROWTHS

3.0
2.0
1.0
0.5
0.3
0.1
1.4
1.0
0.6
80
60
40
40
60
80
100
40
60
80
100

PERIMETER (μm)

Figure 3.38 A group of plasmodesmata between inner secretory cells. Note the clear desmotubules (arrows) and the uniform dimensions of each plasmodesma.

Stimulated tissue - day 4: Spurr's resin: potassium permanganate - lead citrate poststain. x 85,990.

Figure 3.39 A group of plasmodesmata between outer (upper) and inner (lower) secretory cells. Note the continuity between the plasmalemma and the membrane lining the plasmodesmata.

Stimulated tissue - day 4: Spurr's resin: uranyl acetate - lead citrate poststain. x 82,920.

Figure 3.40 A group of plasmodesmata between outer (upper) and inner (lower) secretory cells. Again note the desmotubules (arrows). Note also the unusual branched plasmodesma of the left-hand side.

Stimulated tissue - day 2: Spurr's resin: potassium permanganate - lead citrate poststain. x 80,700.

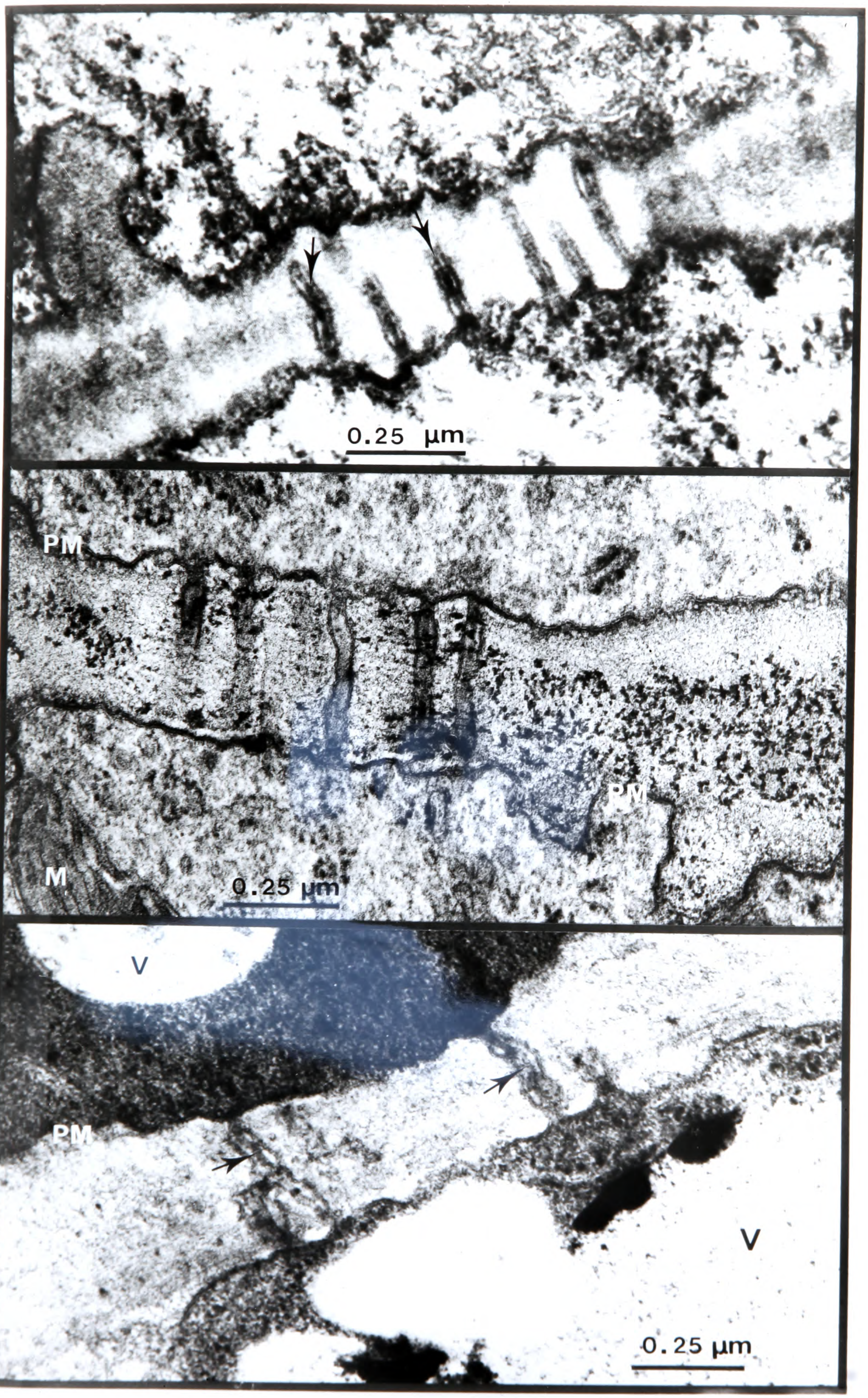

0.25 µm
PM
PM
M
0.25 µm
V
PM
V
0.25 µm

Figure 3.41 Part of the external wall of an outer secretory cell showing the composition of the wall. Note the even texture and continuity of the cuticle. For an explanation of the layers 1 - 6 see text.

Unstimulated tissue: Spurr's resin: lead citrate post-stain. x 39,260.

Figure 3.42 Detail of parts of the external cell walls of an inner secretory cell (upper left) and an epidermal cell (lower right) showing the similarity of their composition. Again note the lack of discontinuities in the cuticle. For an explanation of the layers 1 - 6 see text.

Stimulated tissue - day 2: Spurr's resin: uranyl acetate - lead citrate poststain. x 54,530.

Figure 3.43 A comparison of parts of the cell walls of an inner secretory cell (CW_G) and an epidermal cell (CW_E) showing the difference in thickness. Note in particular the heavy cuticle of the epidermal cell.

Unstimulated tissue: postfixed 10%(w/v) iron II sulphate - 1%(w/v) osmium tetroxide: Spurr's resin: no poststain. x 12,310.

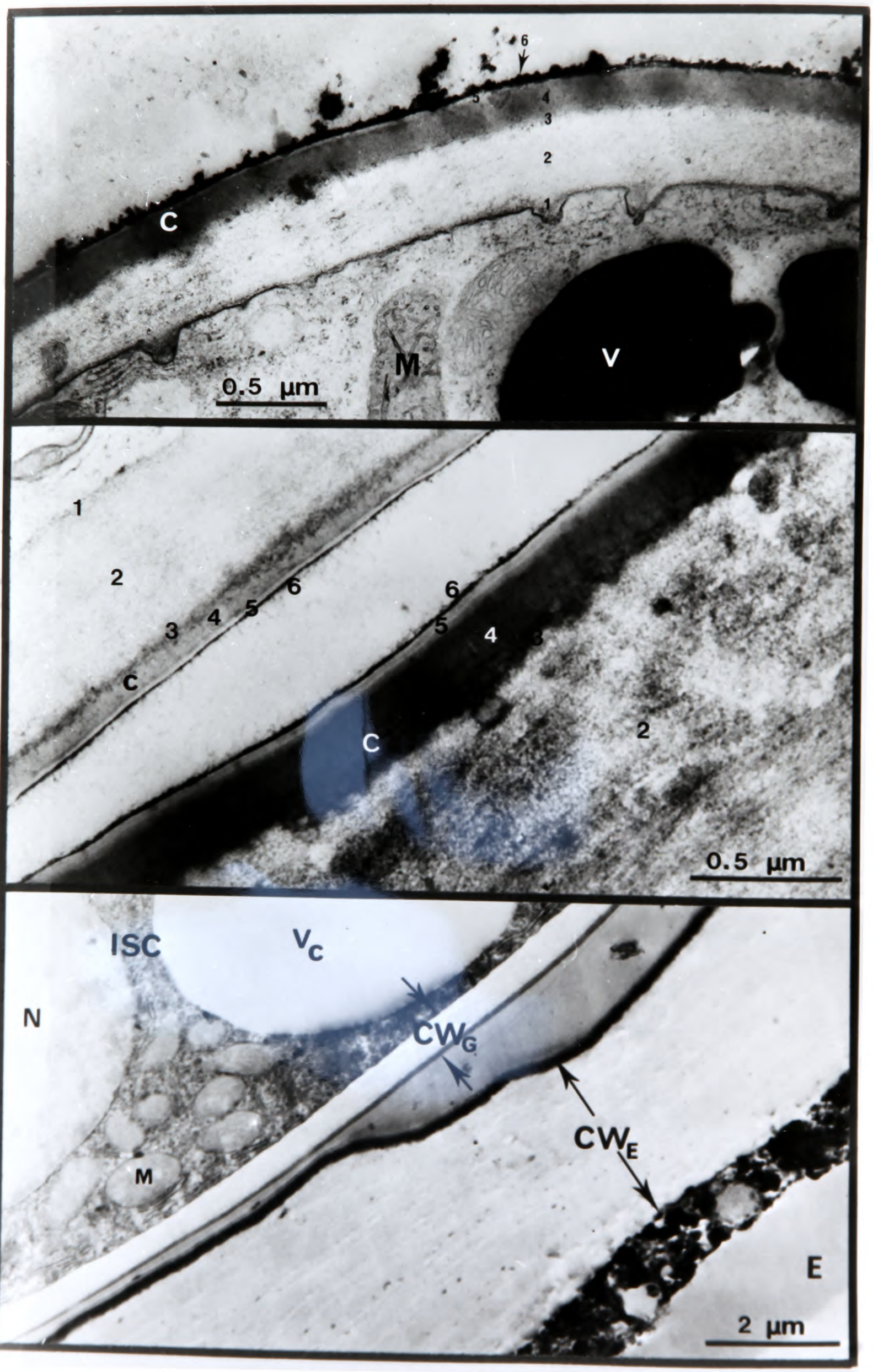

6
5
4
3
2
1
C
M
V
0.5 µm
1
2
3
4
5
6
6
5
4
3
C
C
2
0.5 µm
ISC
V_C
N
CW_G
CW_E
M
E
2 µm

Figure 3.44 Showing the results of the Loud (1962) grid-line quantification of the cytoplasmic compartments of the outer secretory cells.

Each histogram of four blocks displays, from left to right, the % of total cellular volume that is occupied by the cytoplasmic compartment shown on days 0, 2, 4, and 6 after stimulation, except for the endoplasmic reticulum which is expressed as μm per μm^2 of cytosol.

The following changes are shown to be significant by either the t-test or the d-test (Campbell, 1967). Levels of significance defined as in Fig. 3.37.

Compartment	Day versus day	Significance
V_l	0/2	***
	0/6	***
V_c	0/2	**
	0/6	**
V_c	0/4	**
	0/6	**
GP	0/2	***
	4/6	**
N	0/4	**
	4/6	**
M	0/2	***
	4/6	***
P	2/4	**
1^{o}CW	0/2	**
	2/6	**
	4/6	**
LW	0/2	***
	0/4	***
	4/6	**
D	0/2	*
	0/6	*
DV	0/4	*
ER	0/4	**
	0/6	**
	2/4	**
	2/6	***

All other changes over a period of two days or between days 0 and 6 are not significant (NS).

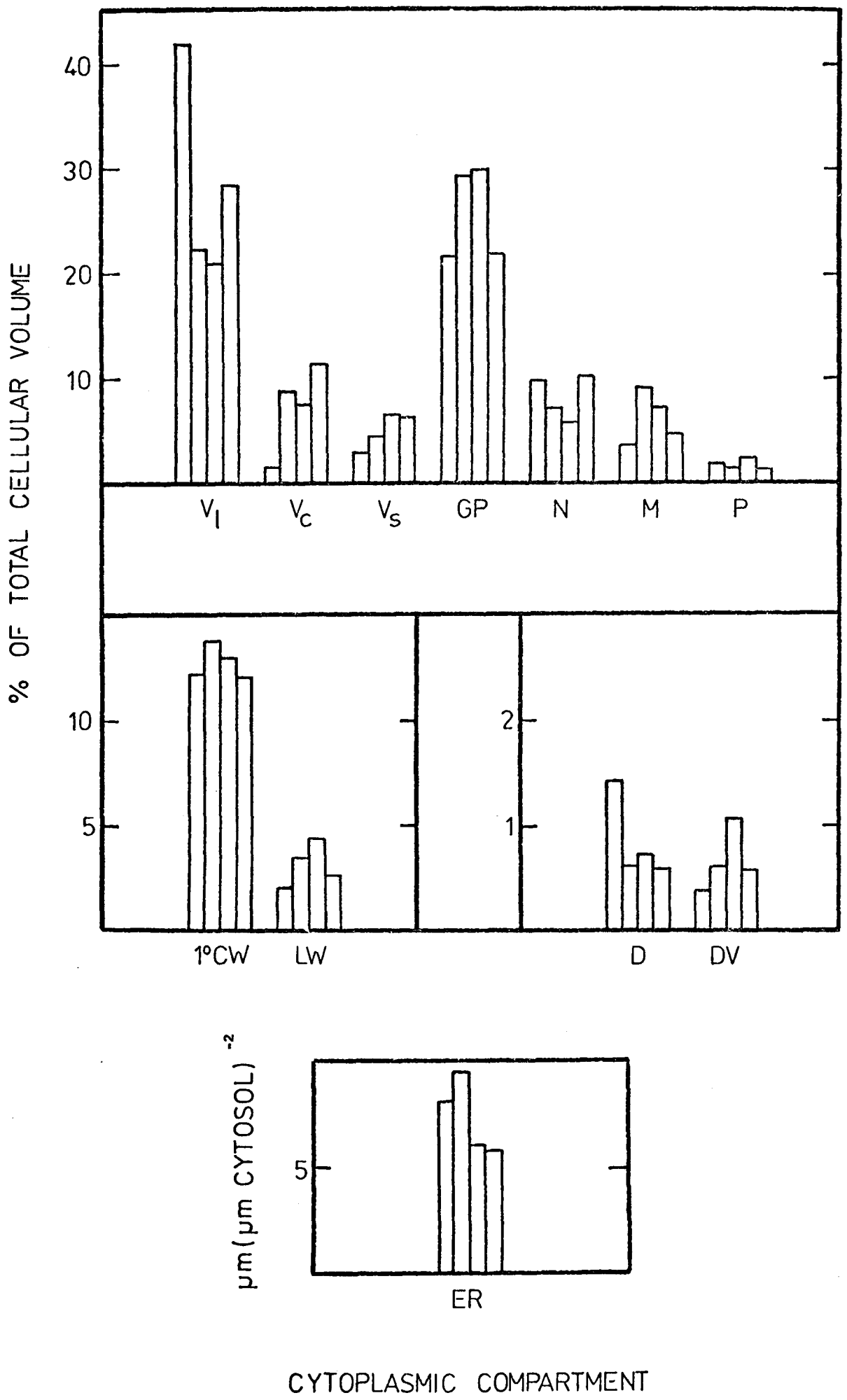
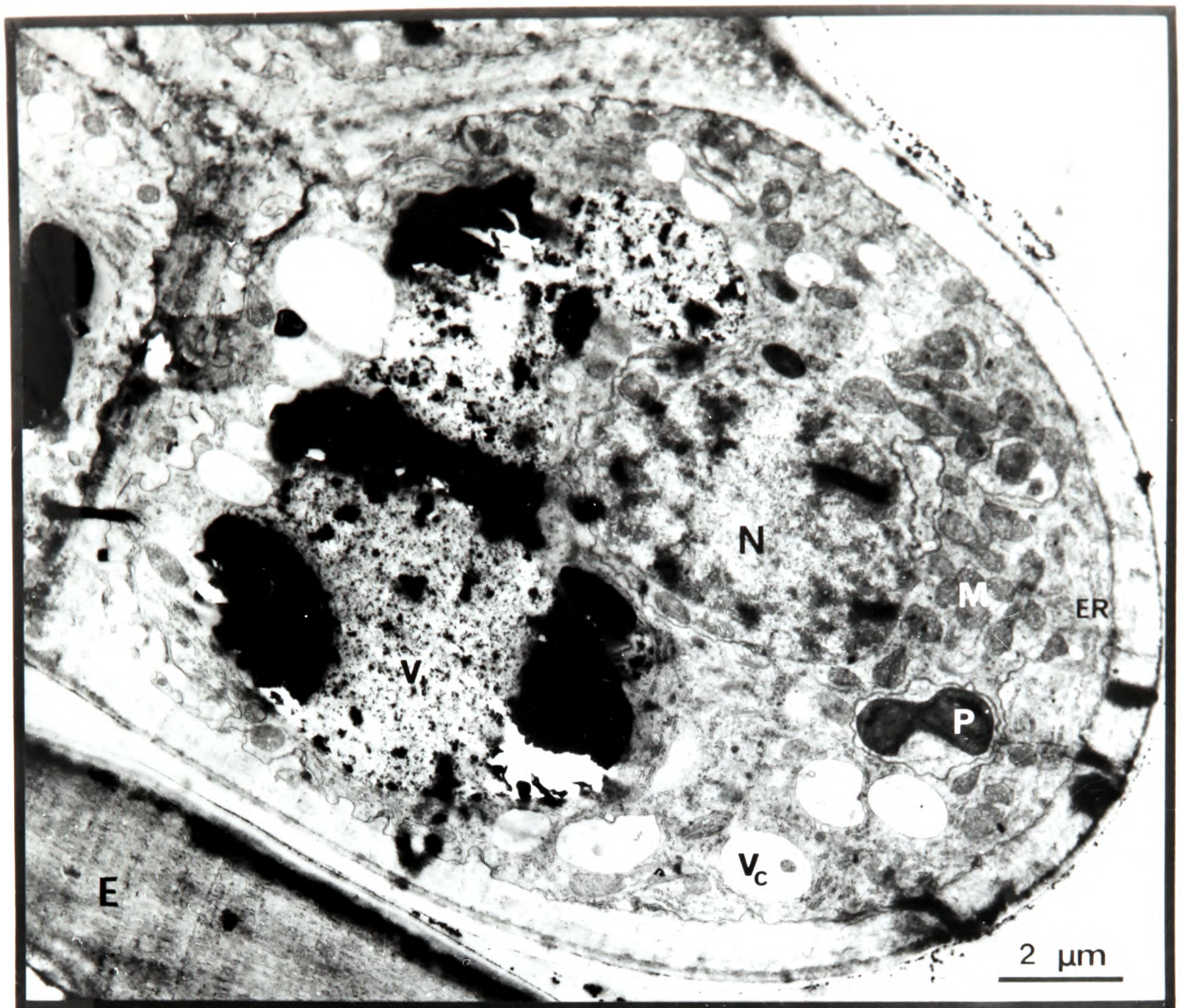

% OF TOTAL CELLULAR VOLUME
40
30
20
10
V_I
V_C
V_S
GP
N
M
P
10
5
2
1
1°CW
LW
D
DV
μm(μm CYTOSOL)$^{-2}$
5
ER
CYTOPLASMIC COMPARTMENT

Figure 3.45 Whole outer secretory cell from two days after stimulation. Note the dispersed appearance of the chromatin.

Stimulated tissue - day 2: Spurr's resin: lead citrate poststain. x 8,470.

Figure 3.46 Whole outer secretory cell from four days after stimulation. Note the numerous clear vacuoles; the high cristae density in the mitochondria; the shapes of many of the mitochondria; and the condensed nature of the chromatin.

Stimulated tissue - day 4: Spurr's resin: uranyl acetate - lead citrate poststain. x 7,600.


N
M
ER
P
V
E
V_C
2 µm

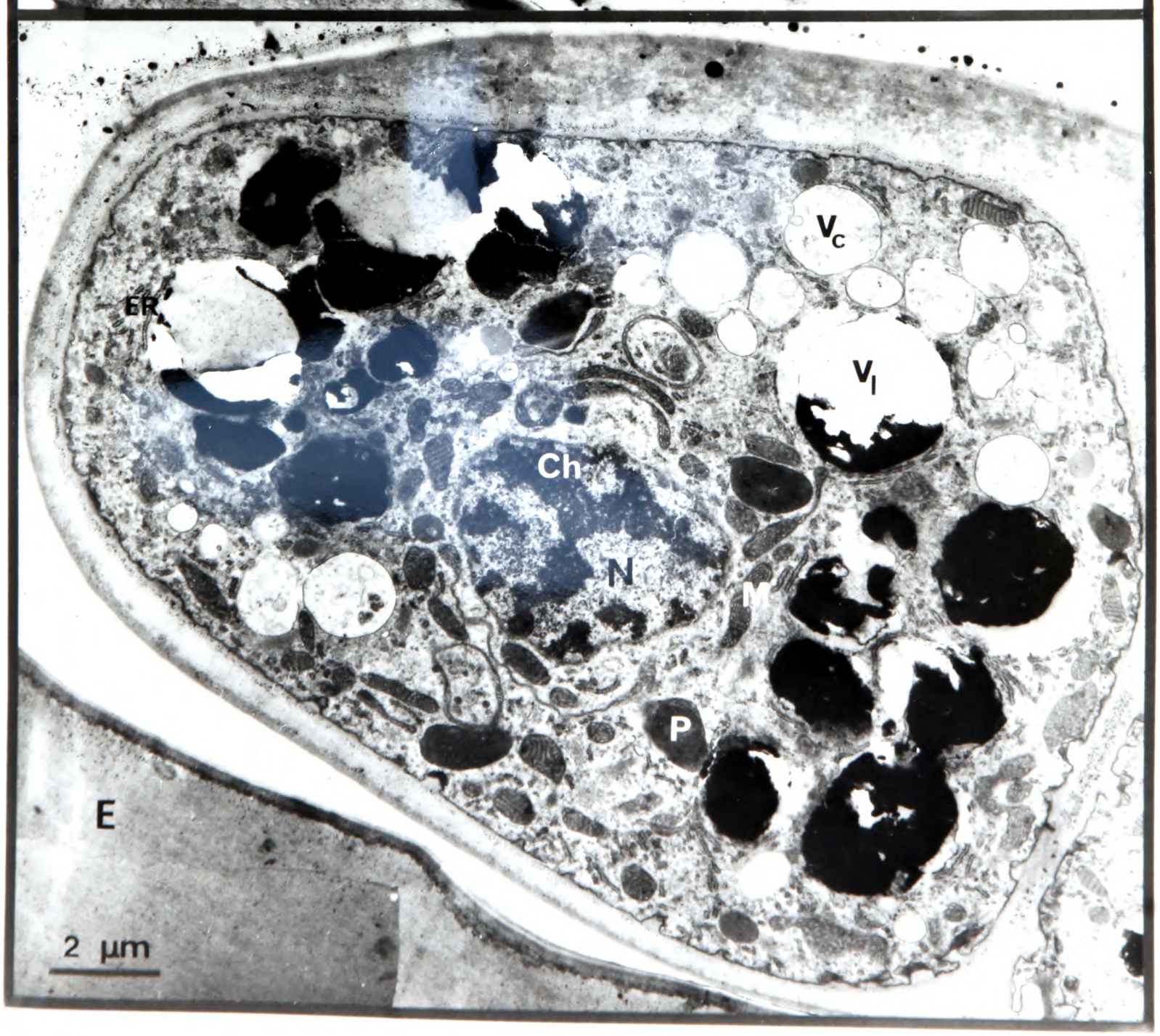

V_C
ER
V_l
Ch
N
M
P
E
2 µm

Figure 3.47 Whole outer secretory cell from six days after stimulation.

Stimulated tissue - day 6: Spurr's resin: lead citrate poststain. x 9,080.

Figure 3.48 Parts of two outer secretory cells showing how radically the fixation of tissue can vary within the same gland. Note how in the upper cell the vacuole is very osmiophilic and the ground-plasm is clear, while in the lower cell the reverse is apparent.

Unstimulated tissue: Spurr's resin: uranyl acetate - lead citrate poststain. x 24,100.

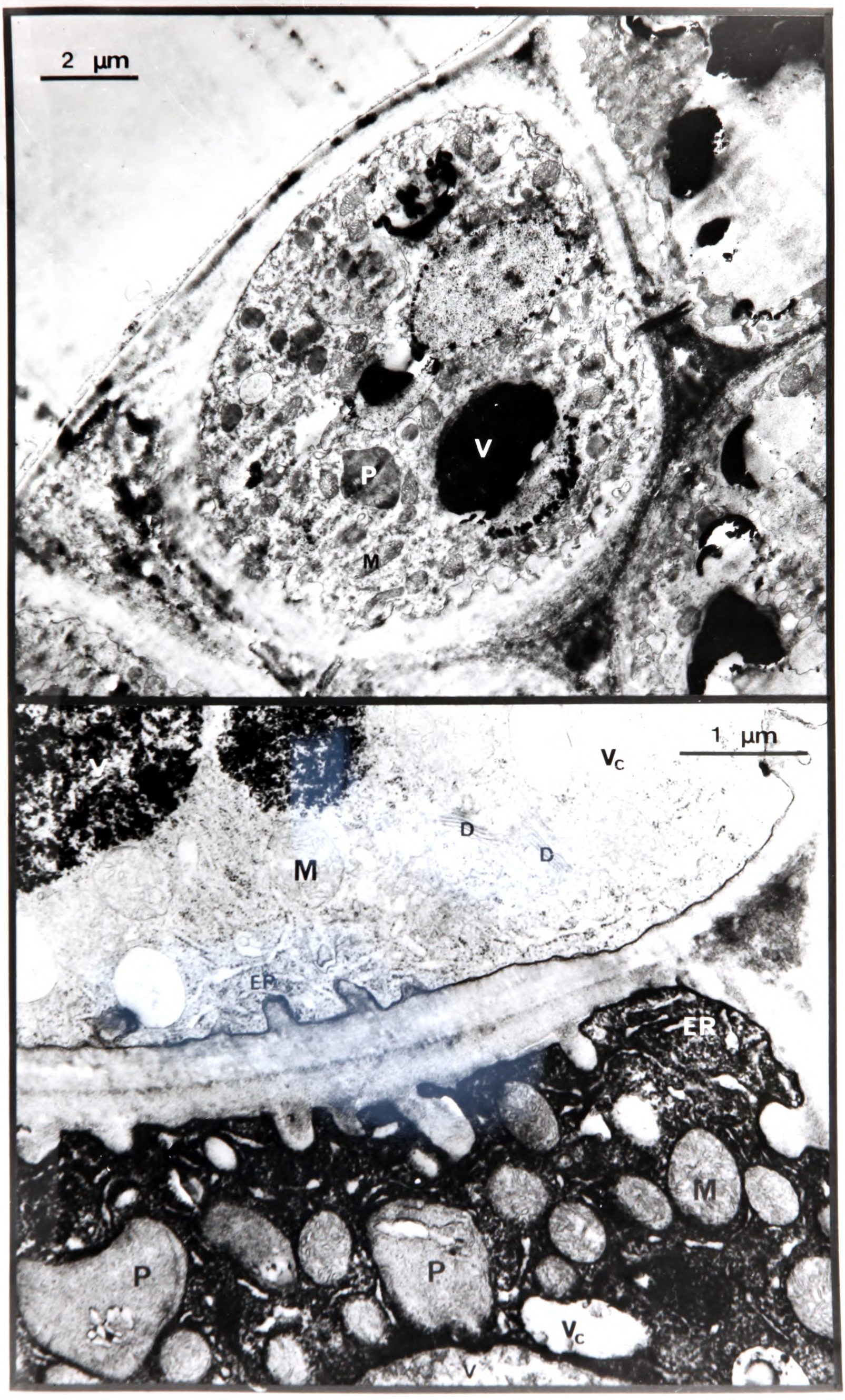

2 µm
V
P
M
1 µm
V
V_C
D
D
M
ER
ER
M
P
P
V_C
V

Figure 3.49 Showing the changes that take place in the mitochondria of the outer secretory cells following stimulation. This figure is taken from Table 3.2, where the details of these mitochondria are given.

a) The mean length of cristae membrane per unit area of mitochondrion;

b) The mean ratio of the length of cristae membrane within a mitochondrion to the perimeter length of that mitochondrion;

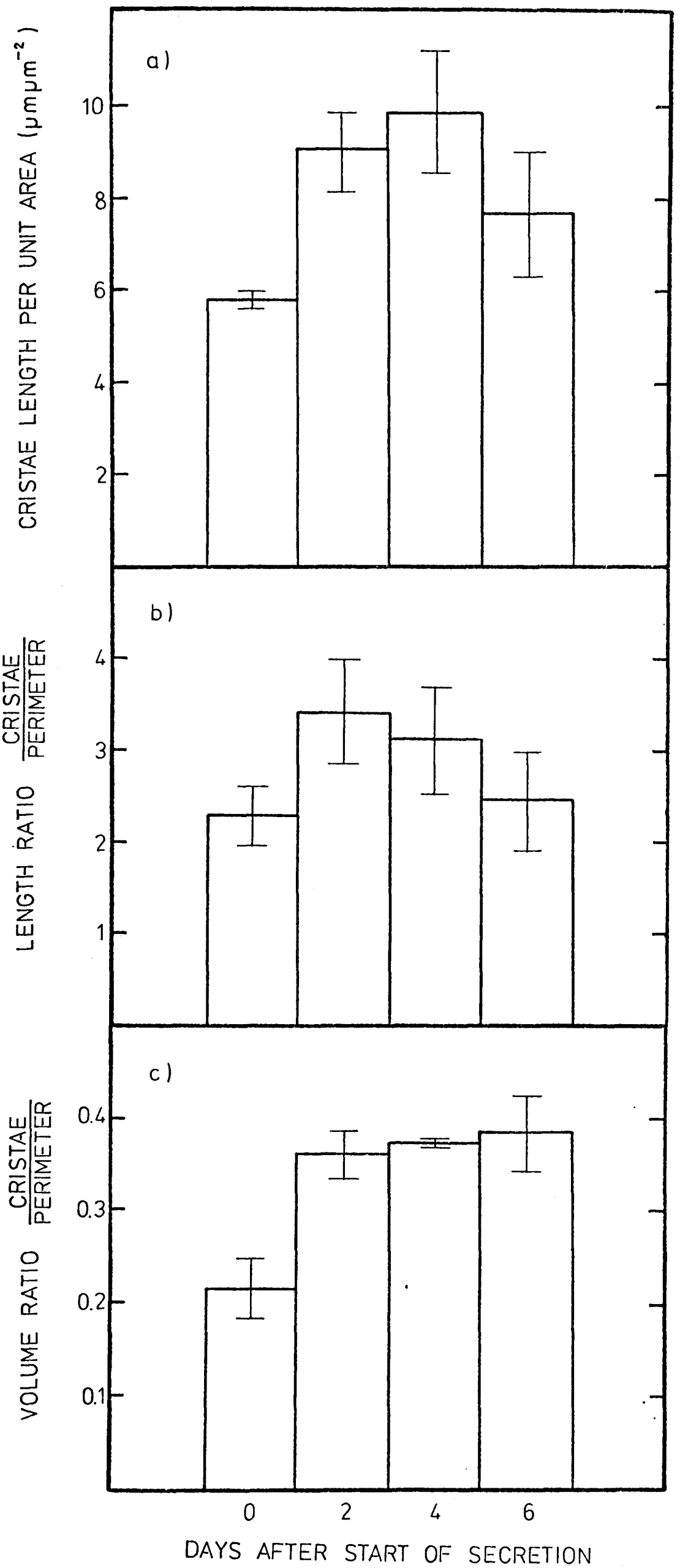
c) The mean ratio of the volume contained within the cristae to the total volume of that mitochondrion.

Histogram values given ± 95% confidence limits.

The following changes are shown to be significant by either the t-test or the d-test (Campbell, 1967). Levels of significance as defined in Fig. 3.37.

	Day versus day	Significance
a)	0/2	***
	4/6	*
b)	0/2	***
	2/6	*
c)	0/2	***
	0/6	***

All other changes over a period of two days or between days 0 and 6 are not significant (NS).


a)
CRISTAE LENGTH PER UNIT AREA (μmμm⁻²)
10
8
6
4
2
b)
LENGTH RATIO CRISTAE / PERIMETER
4
3
2
1
c)
VOLUME RATIO CRISTAE / PERIMETER
0.4
0.3
0.2
0.1
0
2
4
6
DAYS AFTER START OF SECRETION

Figures 3.50 - 3.53 Mitochondria from outer secretory cells at different stages of the secretory cycle:

Fig. 3.50

Unstimulated tissue - day 0.

x 103,600.

Fig. 3.51

Stimulated tissue - day 2.

x 86,400.

Fig. 3.52

Stimulated tissue - day 4.

x 109,200.

Fig. 3.53

Stimulated tissue - day 6.

x 74,400.

All tissue from the same preparation: Spurr's resin: uranyl acetate - lead citrate poststain.

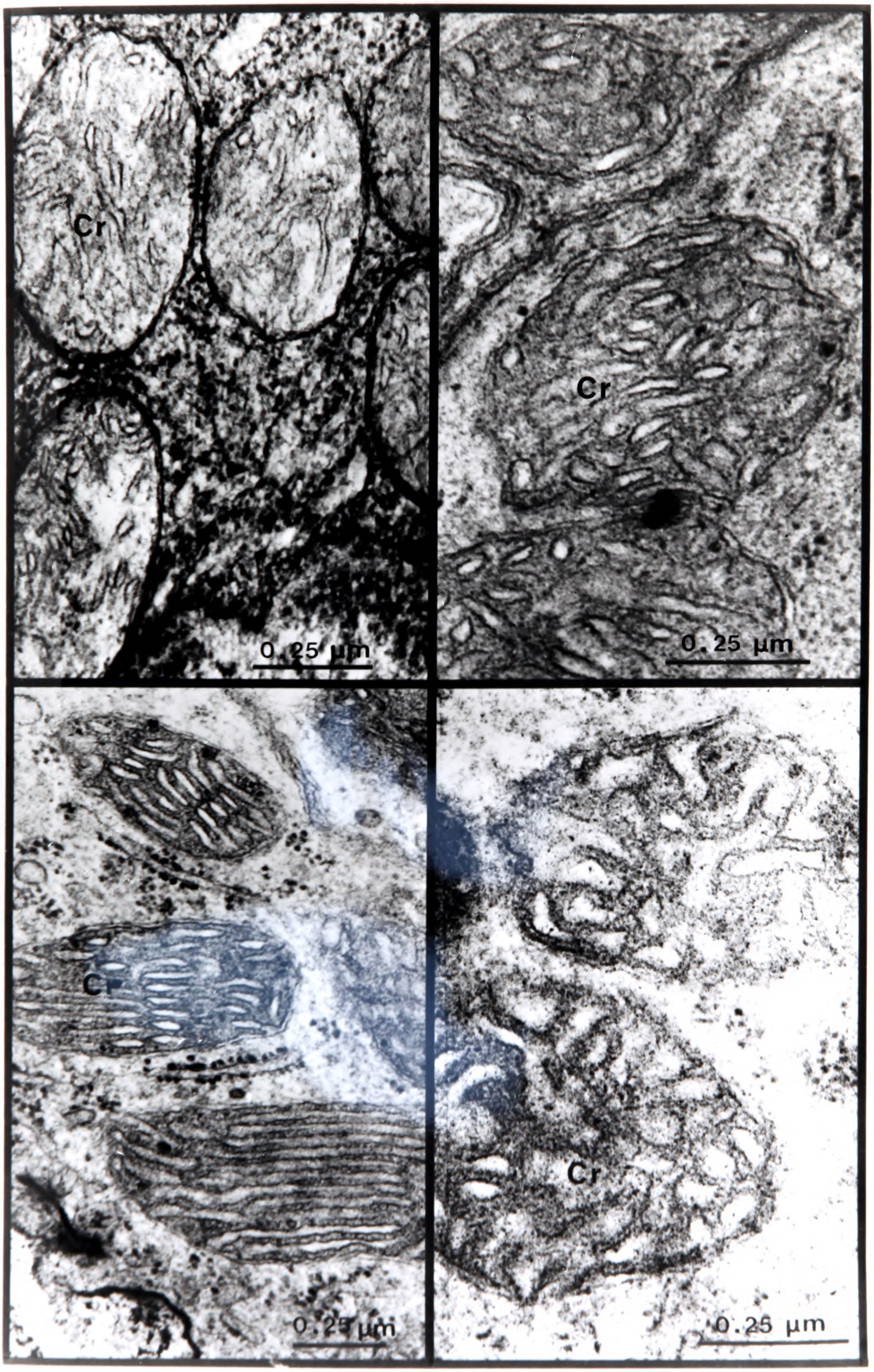

Cr
0.25 µm
Cr
0.25 µm
Cr
0.25 µm
Cr
0.25 µm

Figure 3.54 Detail from an outer secretory cell showing unusual, extremely elongated mitochondria. Note how all the ribosomes are associated with endoplasmic reticulum except for a few on the nuclear envelope.

Stimulated tissue - day 2: Spurr's resin: uranyl acetate lead citrate poststain. x 41,090.

Figure 3.55 Detail from an outer secretory cell showing further unusual mitochondrial sections.

Stimulated tissue - day 4: Spurr's resin: uranyl acetate lead citrate poststain. x 42,700.

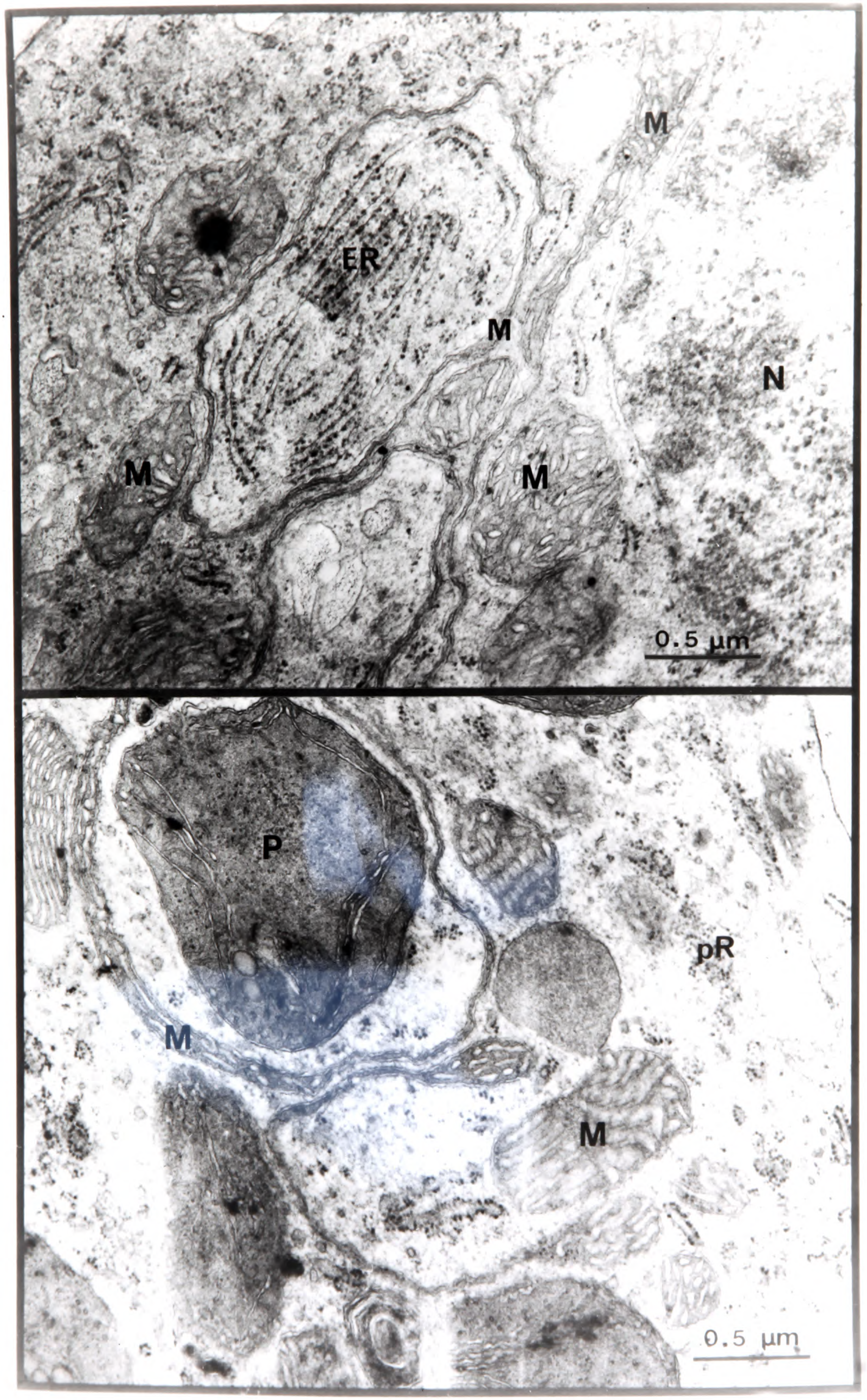

M
ER
M
N
M
M
0.5 µm
P
pR
M
M
0.5 µm

Figure 3.56 Detail from an outer secretory cell showing endoplasmic reticulum with associated polysomes.

Stimulated tissue - day 4: Spurr's resin: uranyl acetate - lead citrate poststain. x 61,820.

Figure 3.57 Detail from an outer secretory cell showing endoplasmic reticulum with a typical, very dense population of membrane-bound ribosomes.

Stimulated tissue - day 4: Spurr's resin: uranyl acetate - lead citrate poststain. x 113,600.

Figure 3.58 Detail from the outer edge of an outer secretory cell showing a profile of endoplasmic reticulum fused to the plasmalemma.

Stimulated tissue - day 4: Spurr's resin: potassium permanganate - lead citrate poststain. x 114,400.

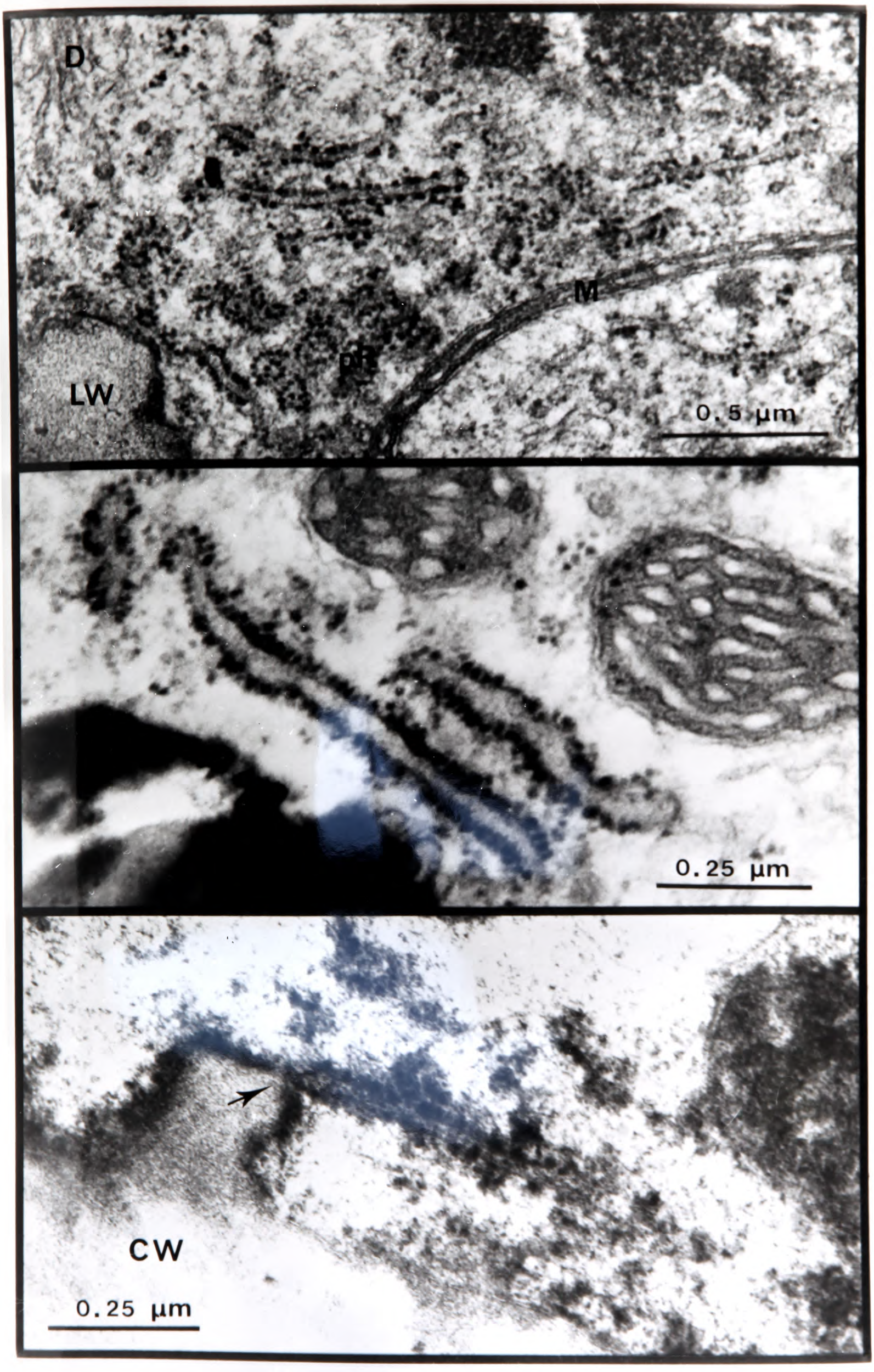
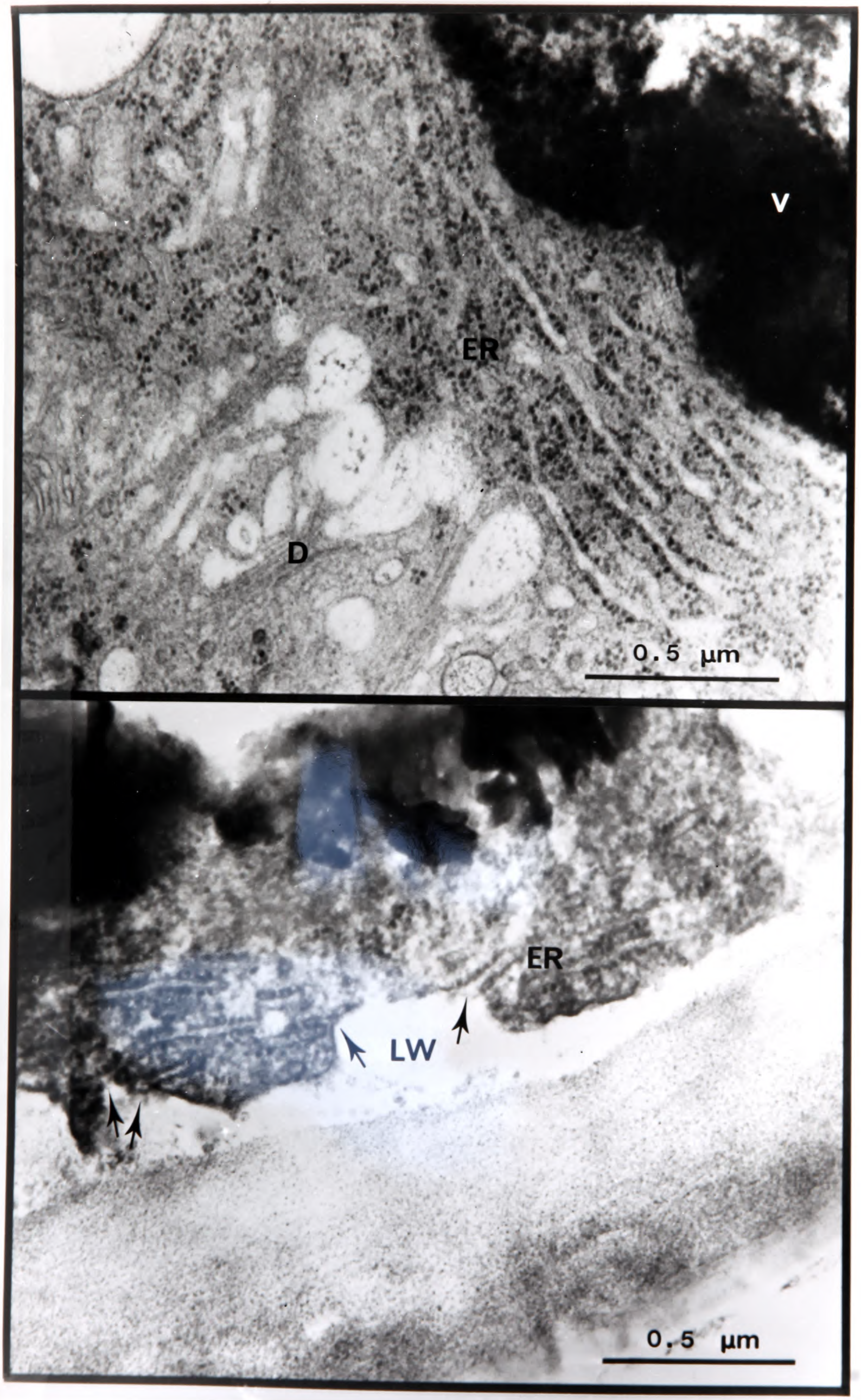

D
M
PR
LW
0.5 µm
0.25 µm
CW
0.25 µm

Figure 3.59 Detail from an outer secretory cell showing stacked endoplasmic reticulum next to a large osmiophilic vacuole. Note the active dictyosome, in contrast to Fig. 3.56.

Stimulated tissue - day 2: Spurr's resin: uranyl acetate - lead citrate poststain. x 72,310.

Figure 3.60 Detail from the edge of an outer secretory cell showing profiles of rough endoplasmic reticulum fused to the plasmalemma at points of labyrinthine cell wall. Note the numerous associated ribosomes.

Stimulated tissue - day 4: Spurr's resin: uranyl acetate - lead citrate poststain. x 71,650.


V
ER
D
0.5 µm
ER
LW
0,5 µm

Figures 3.61 and 3.62 Two photo-micrographs showing adjacent regions of the external edge of an outer secretory cell. Note how profiles of endoplasmic reticulum are lying tightly apposed to the plasmalemma, with ribosomes bound only to the cytoplasmic face. Note also how some of these profiles are apparently fused with the plasmalemma (arrows) at points of invagination, which contain small vesicles and are probably plasmalemmasomes.

Stimulated tissue - day 4: Spurr's resin: lead citrate poststain.

Fig. 3.61 x 69,600

Fig. 3.62 x 70,580

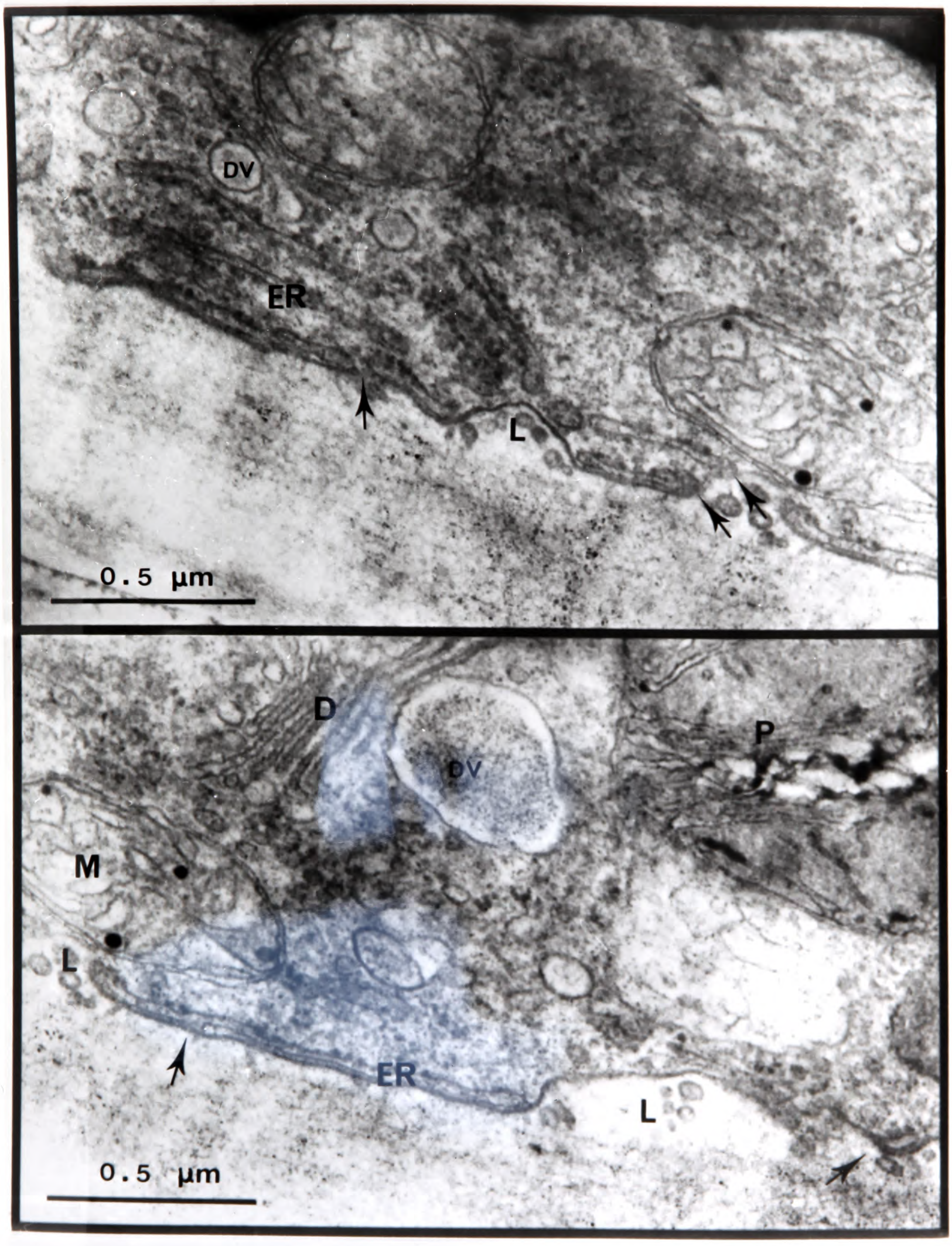

DV
ER
L
0.5 µm
D
DV
P
M
L
ER
L
0.5 µm

Figure 3.63 Detail from an outer secretory cell showing polysomes apparently overlying a region of endoplasmic reticulum.

Stimulated tissue - day 2: Spurr's resin: uranyl acetate - lead citrate poststain. x 123,200.

Figure 3.64 Detail from an outer secretory cell showing a dense concentration of polysomes apparently associated with underlying endoplasmic reticulum.

Stimulated tissue - day 4: Spurr's resin: uranyl acetate - lead citrate poststain. x 70,830.

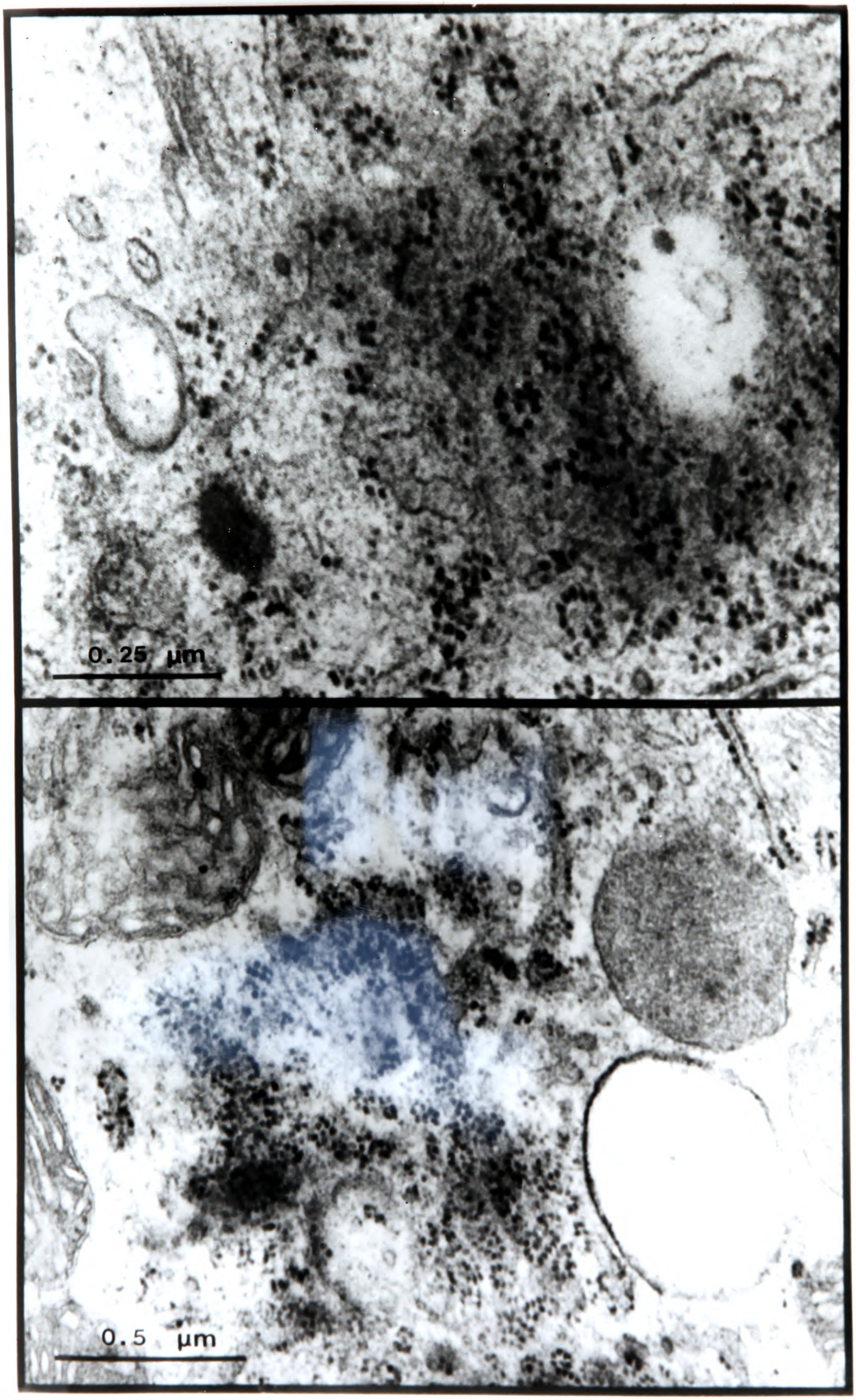

0.25 µm
0.5 µm

Figure 3.65 Detail of the cytoplasm of an outer secretory cell adjacent to the cell periphery. Showing rough endoplasmic reticulum undergoing vesiculation and swelling. Note how the points of fusion and fission are devoid of ribosomes.

Stimulated tissue - day 4: Spurr's resin: uranyl acetate - lead citrate poststain. x 59,530.

Figure 3.66 Detail from the cytoplasm of another outer secretory cell adjacent to the cell periphery showing rough endoplasmic reticulum undergoing vesiculation and swelling. Note especially how it appears that vesicles are migrating between the rough endoplasmic reticulum and a clear vacuole.

Stimulated tissue - day 4: uranyl acetate - lead citrate poststain. x 78,730.

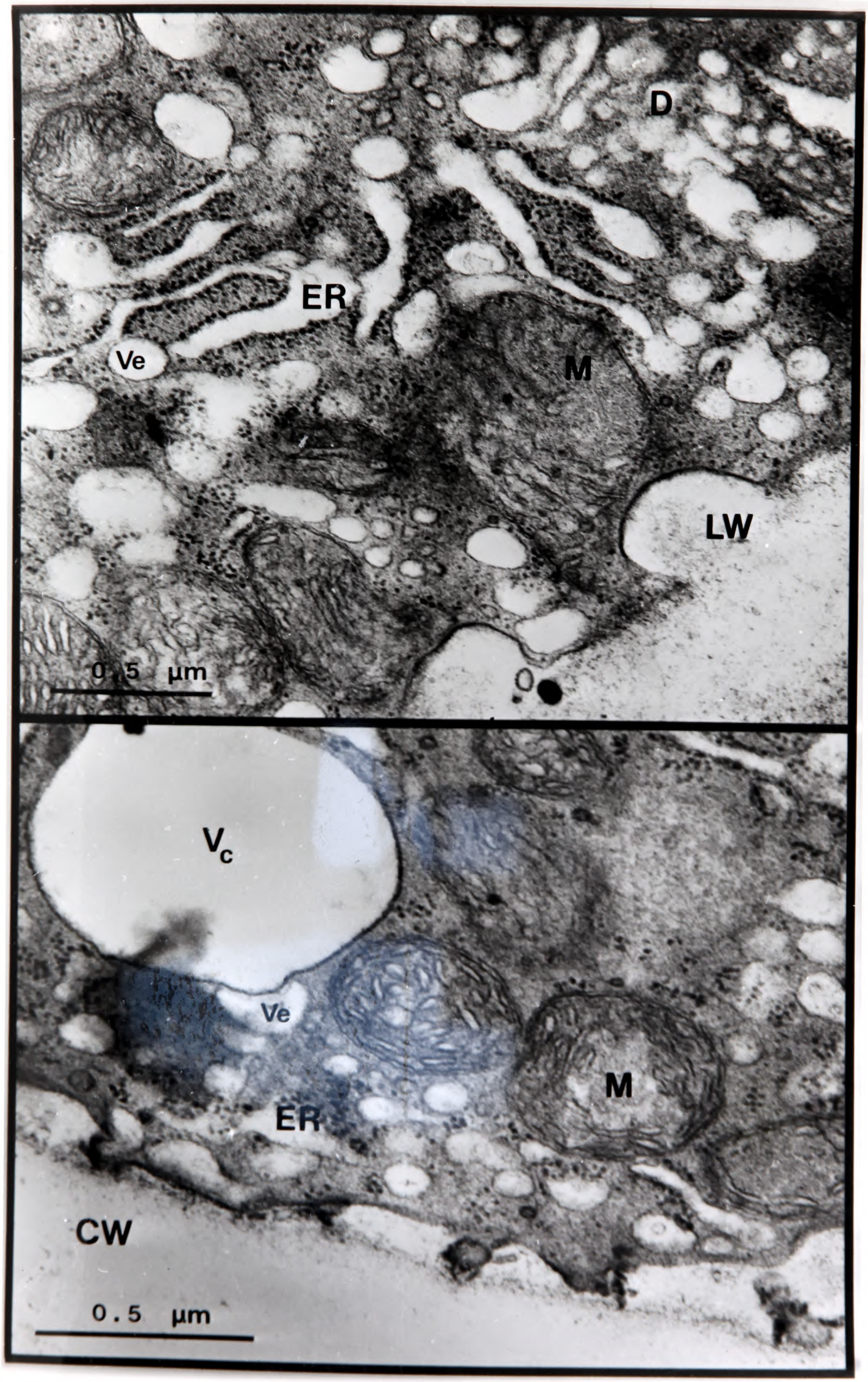

D
ER
Ve
M
LW
0.5 µm
Vc
Ve
M
ER
CW
0.5 µm

Figure 3.67 Detail from the cytoplasm of an outer secretory cell showing distended profiles of rough endoplasmic reticulum which have a high density of ribosomes throughout much of the surface.

Stimulated tissue - day 6: Spurr's resin: lead citrate poststain. x 57,010.

Figure 3.68 Part of the cytoplasm of an outer secretory cell adjacent to the cell periphery showing vesicles, derived from a dictyosome, migrating to the plasmalemma. The vesicles show two distinct types of content.

Stimulated tissue - day 2: Spurr's resin: lead citrate poststain. x 67,130.

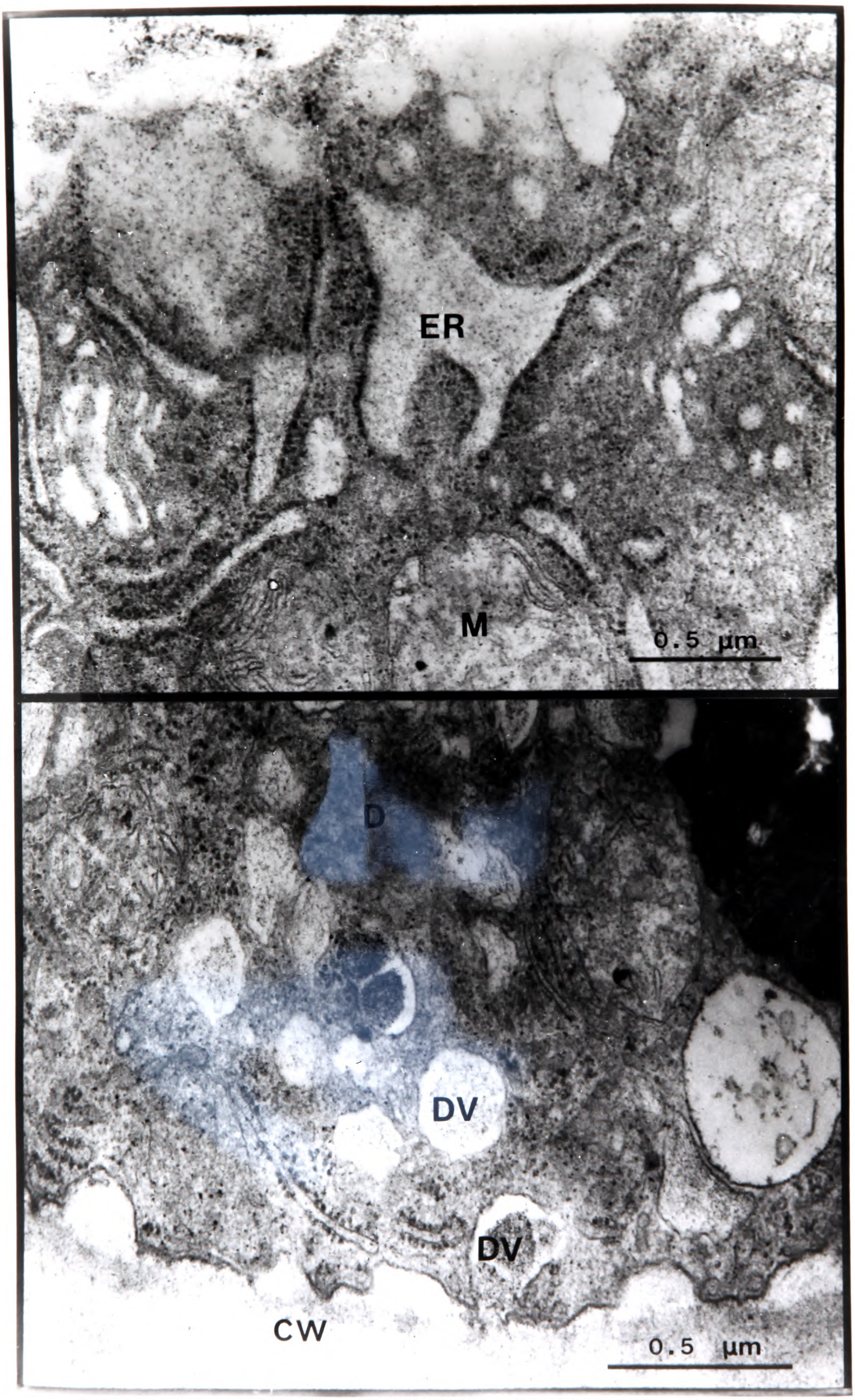

ER
M
0.5 µm
D
DV
DV
CW
0.5 µm

Figure 3.69 Detail from the cytoplasm of an outer gland cell showing vesicles with granular contents fusing to the plasmalemma. They do not appear to arise from the dictyosome seen in this figure.

Stimulated tissue - day 2: Spurr's resin: lead citrate poststain. x 80,210.

Figure 3.70 Dictyosomes in an outer secretory cell showing vesicles which have no permanganophilic contents contrasting with the cisternal membranes.

Stimulated tissue - day 2: Spurr's resin: potassium permanganate - lead citrate poststain. x 75,770.

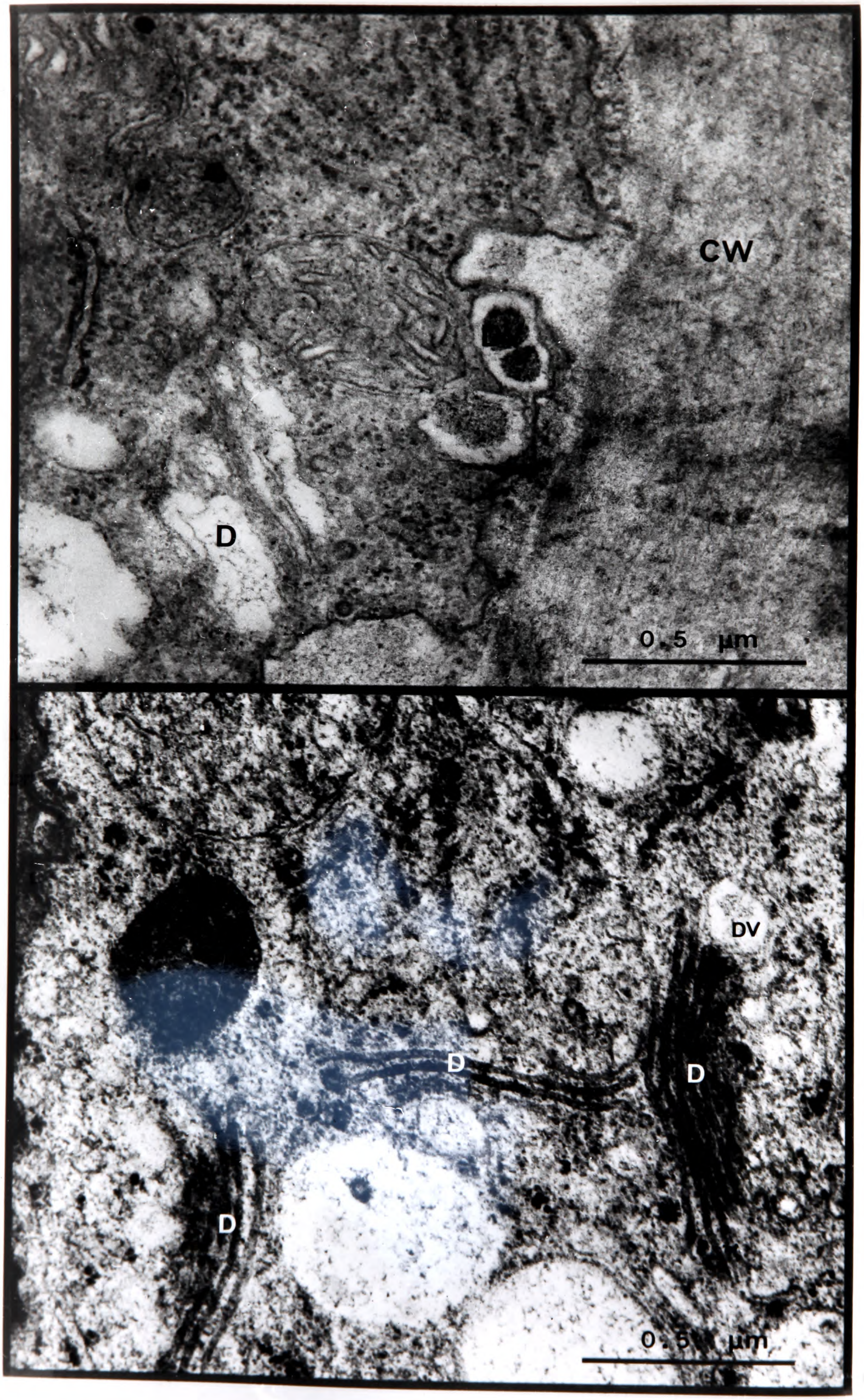

CW
D
0.5 µm
DV
D
D
D
0.5 µm

Figure 3.71 Detail from the cytoplasm of an inner secretory cell showing a lomasome close to the plasmalemma.

Stimulated tissue - day 2: Spurr's resin: lead citrate poststain. x 78,480.

Figure 3.72 Detail from the cytoplasm at the periphery of an outer secretory cell showing a lomasome close to the plasmalemma.

Stimulated tissue - day 4: Spurr's resin: lead citrate poststain. x 113,60.

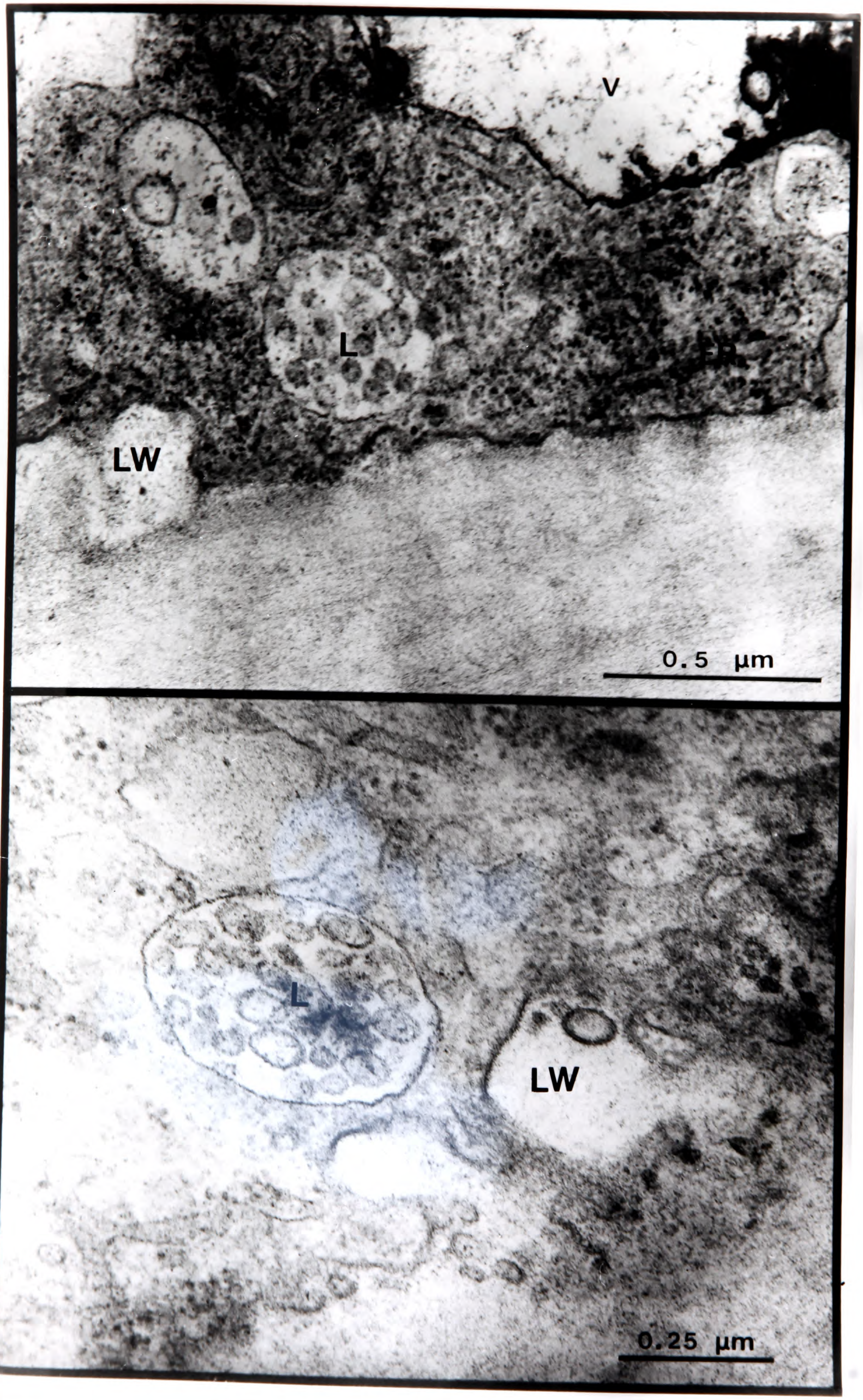

V
L
ER
LW
0.5 µm
L
LW
0.25 µm

Figure 3.73 Showing the amplification factor at various boundaries within the gland in lobes sampled 0, 2, 4, and 6 days after the start of secretion.

a) Outer secretory cell - whole perimeter;

b) Inner secretory cell - whole perimeter;

c) Outer secretory cell/exterior;

d) Outer secretory cell/outer secretory cell;

e) Outer secretory cell/inner secretory cell;

f) Inner secretory cell/exterior;

g) Inner secretory cell/outer secretory cell;

h) Inner secretory cell/inner secretory cell;

i) Inner secretory cell/stalk cell.

The following changes are shown to be significant by either the t-test or the d-test (Campbell, 1967). Levels of significance as defined in Fig. 3.37.

	Day versus day	Significance
b)	4/6	**
d)	4/6	**
f)	0/4	*
	2/4	*
g)	0/4	**

All other changes are not significant (NS).

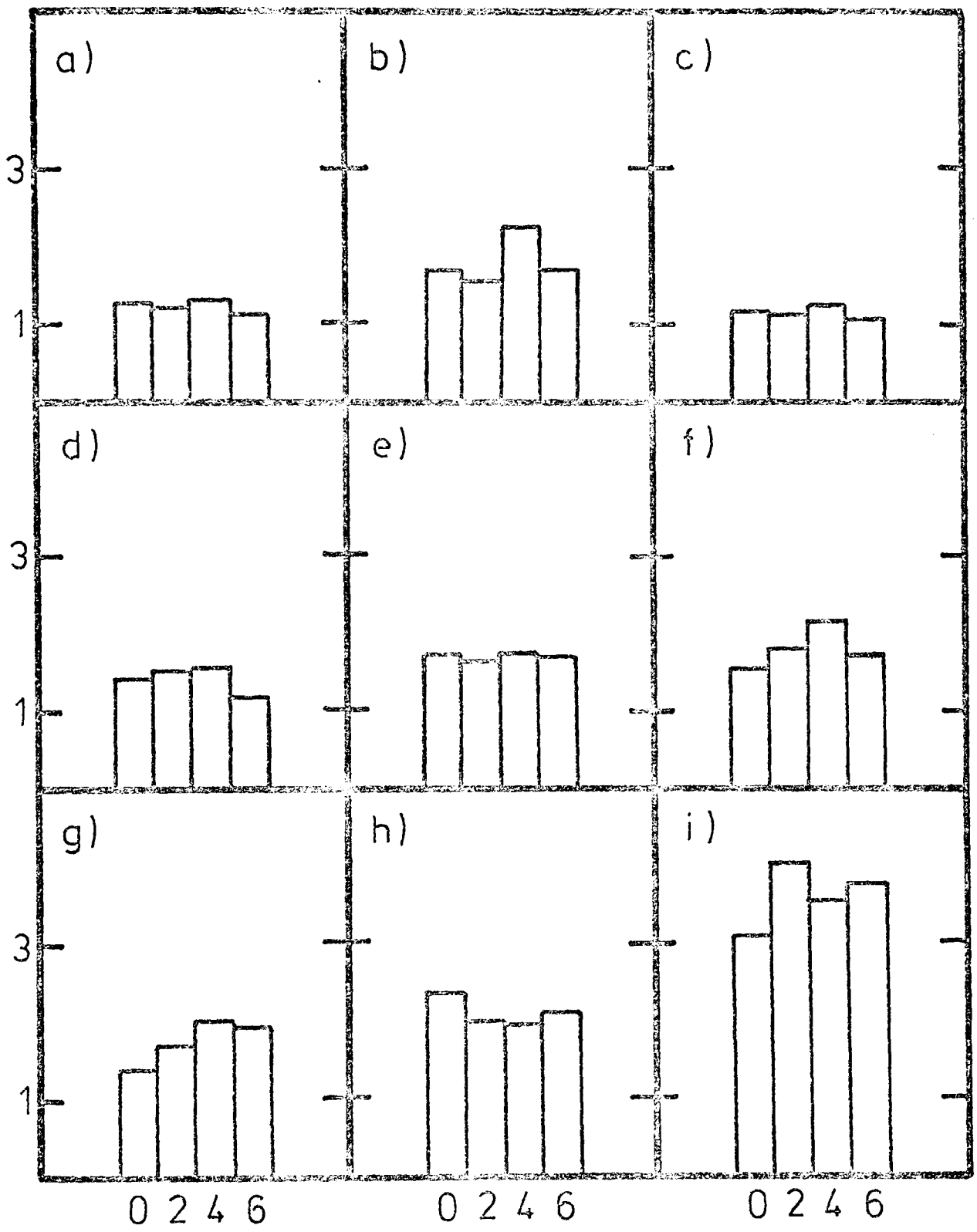

a)
b)
c)
d)
e)
f)
g)
h)
i)
3
1
3
1
3
1
0 2 4 6
0 2 4 6
0 2 4 6
AMPLIFICATION FACTOR
DAYS AFTER START OF SECRETION

Figure 3.74 Detail from the cytoplasm of a stalk cell showing the overall decrease in contrast in the contents of the lipid droplets after prolonged secretion. Compare with Figs. 3.10 and 3.11.

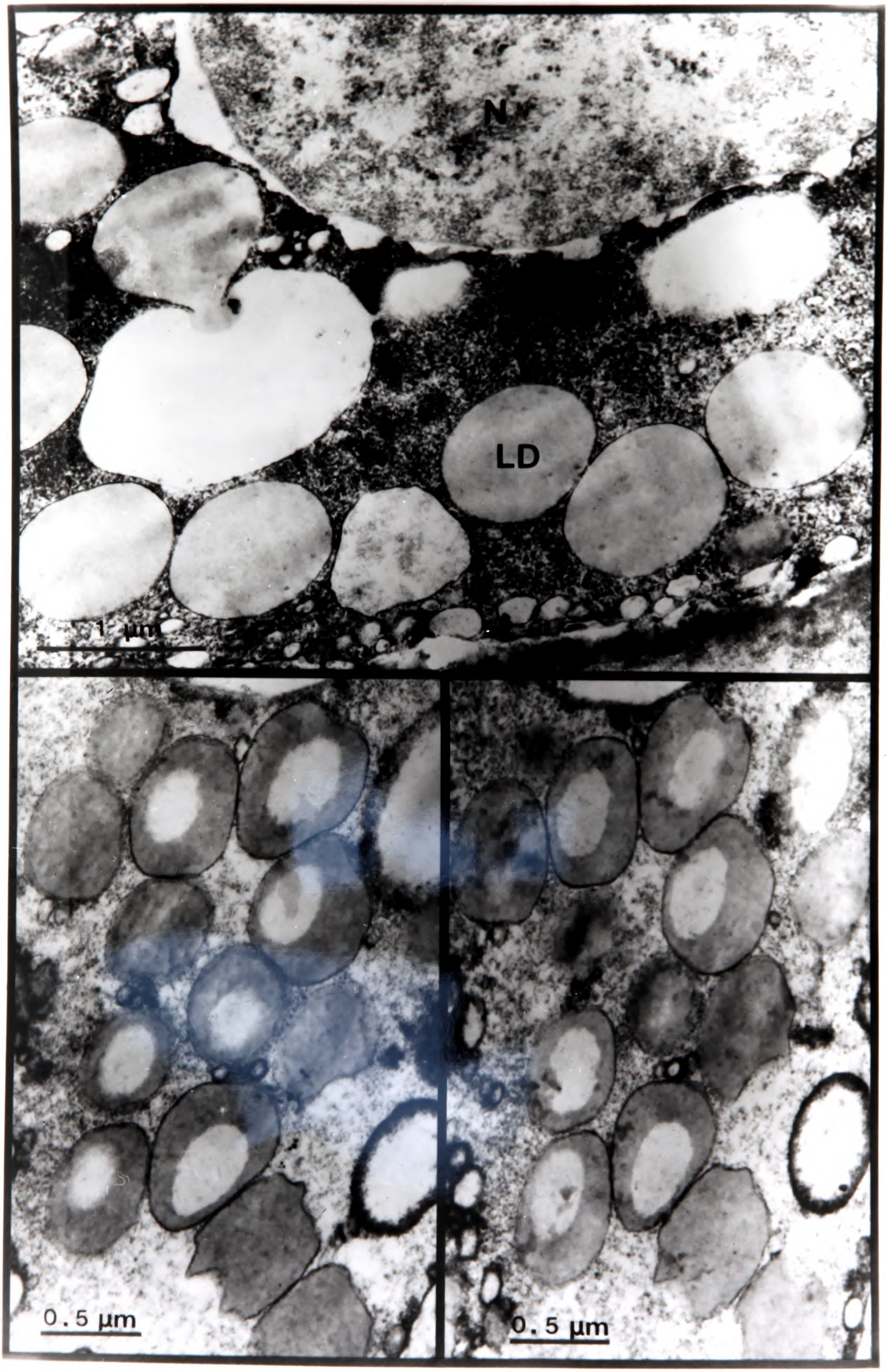
Stimulated tissue - day 6: Spurr's resin: lead citrate poststain. x 31,100.

Figures 3.75 and 3.76 show lipid droplets in a stalk cell from two adjacent sections. They demonstrate that the depletion of material unevenly throughout the droplets is not due to a thin-section artefact.

Stimulated tissue - day 6: Spurr's resin: uranyl acetate - lead citrate poststain.

Fig. 3.75 x 36,940.

Fig. 3.76 x 36,270.


N
LD
1 µm
0.5 µm
0.5 µm

CHAPTER 4

SOURCE OF THE SECRETED PROTEIN

I INTRODUCTION

II RESULTS

1) Localization of Acid β-glycerophosphatase and ATPase by the Gomori Reaction

2) Mercury II - Bromophenol Blue Treatment

3) Thin-Section Digestion with Pronase and Lipase

4) Iron II Sulphate Treatment

5) Autoradiography

6) Density Labelling

7) Radiolabelling

III DISCUSSION

1) Storage of the Secreted Proteins

2) Synthesis of the Secreted Proteins

I INTRODUCTION

In previous work (Schwab et al, 1969) it was suggested that the protein secreted by Dionaea was stored, prior to stimulation, in the large osmiophilic vacuoles of the secretory cells. This conclusion was partially based on the observed histochemical staining properties of the vacuoles in the light microscope, where both a ninhydrin-positive and a Sudan III-positive reaction was obtained. The extent of the staining was found to decrease considerably as a result of stimulation, but to return to the initial intensity by the end of the cycle. They therefore suggested that the changes in the vacuoles, discussed in the previous chapter, reflect the release of hydrolytic enzymes from these organelles and that this supply is regenerated by protein synthesis towards the end of the cycle.

This chapter will analyse the results of experiments conducted to examine these factors. These entailed (a) high resolution cytochemical, histochemical and autoradiographic work in the electron microscope and (b) radio- and heavy-isotope labelling experiments to look for protein synthesis.

Cytochemical work on the hydrolases of insectivorous plants is scarce and only Vintéjoux, working with Utricularia (Vintéjoux, 1970b; 1973c; 1974a, b) has examined this aspect at the electron microscope level. The earliestwork is that of Palczewska (1966) who showed protease to be released from the stalked glands of Drosera rotundifolia by using the gelatin-film substrate method of Adams and Tuqan (1961). The

most detailed individual analysis is that of Heslop-Harrison and Knox (1971) on Pinguicula, while Heslop-Harrison (1975; 1976) presents comprehensive reviews to date of the literature and includes some otherwise unpublished work of her own on other genera.

II RESULTS

1) Localization of Acid β-glycerophosphatase and ATPase by the Gomori Reaction

Acid β-glycerophosphatase was chosen as a marker for the secretion as Scala et al (1969) had previously shown acid phosphatase (p-nitrophenylphosphate hydrolase) present at high activity in the secretion, and this is confirmed in this study. Sexton et al (1971) examined a number of conditions in order to obtain a satisfactory reaction and concluded that the use of very small pieces of tissue combined with a short period of fixation in the cold gave the most satisfactory activity. Sodium β-glycerophosphate is commonly used as the substrate, as it is readily taken into cells.

Small deposits of lead II phosphate are numerous in the primary cell wall after 30 - 60 min incubation of tissue with substrate. In the internal walls they are particularly dense along the middle lamella (Fig. 4.5). They are, however, conspicuously absent from the labyrinthine wall (Figs. 4.1 : 4.2), the cuticularized part of the external wall (Fig. 4.1) and the epidermal cell wall (Fig. 4.2). Control material shows no such deposits (Figs. 4.3 : 4.4).

A heavy, continuous deposit occurs within the tonoplasts of the large vacuoles (Figs. 4.1 : 4.2). The vacuolar deposition occurs in tissue which has not been post-fixed with osmium tetroxide (Fig. 4.2), is absent from osmicated controls in this preparation (Figs. 4.3 : 4.4), and hence is due to phosphatase activity. No activity is detected in controls of tissue boiled for 15 min before incubation.

Some activity is present associated with the plasmalemma and endoplasmic reticulum (Fig. 4.2) but no activity is found associated with dictyosomes.

In contrast, material incubated 90 - 180 min for ATPase activity shows no activity in the primary wall but considerable deposition of lead II phosphate in the labyrinthine wall (Figs. 4.6 : 4.7). Although some of these deposits are in the matrix of the wall, they are principally associated with either the plasmalemma or the labyrinthine/primary wall interface (Fig. 4.7). In one preparation, where extensive lysis occurs after 180 min incubation, there is a strong deposit of reaction product along the inner face of the plasmalemma, which has become detached from the cell wall and is free in the evacuated cell space (Fig. 4.11).

Hydrolysis product is not noticeably present along the tonoplast, there being no more density here than in the controls (Fig. 4.8 : 4.9). There are, however, small deposits scattered throughout the cytoplasm, notably associated with mitochondria and endoplasmic reticulum (Figs. 4.6 : 4.7) which are absent from the controls (Figs. 4.8 : 4.9).

2) Mercury II Chloride - Bromophenol Blue Treatment

This stain, used for some time in the light microscope (Johansen, 1940), has been adapted to the electron microscope where the mercury may be used to introduce electron density. Fig. 4.12 shows fresh material stained for 20 min for proteins by this method. The staining of the glandular

secretory cells is intense compared to the underlying tissue, and the vacuoles of some cells are particularly dense.

Material fixed and incubated for 90 min in mercury II - bromophenol blue solution (Figs. 4.13 - 4.15) shows heavy staining in all the vacuolar compartments which is absent from the controls. The nucleus, mitochondria and ground-plasm all stain intensely, whereas the endoplasmic reticulum does not. The labyrinthine wall remains transparent except for long fibrillar regions leading from the protruberances to the primary wall matrix and presumably consisting of the core material (see Fig. 3.36). The primary cell wall stains a little more intensely than in material treated with glutaraldehyde only and the cuticle similarly shows no electron density at all. The outermost wall layer, however, is darkly stained.

3) Thin-Section Digestion with Pronase and Lipase

Pronase treatment for 90 min has little effect on either the structural integrity of the cytoplasm (Fig. 4.16) or the droplets in the stalk cells (Fig. 4.17). The osmio-philic vacuoles, however, show a marked, but incomplete, loss of contrast relative to the rest of the cytoplasm (Figs. 4.18 : 4.19). Lipase has no effect on the osmiophilic vacuoles (Fig. 4.20) while a mixture of pronase and lipase has no greater effect upon them than pronase itself (Fig. 4.21).

4) Iron II Sulphate Treatment

Lipase and pronase treatments do not, either separately or together, remove the majority of the osmiophilic material from the main vacuoles of the gland-cells. The osmiophilic affinity of these organelles is very high, and it was considered likely that this may be due to either the cyanidin-3-glucoside present (Di Gregorio and Di Palma, 1966) or phenols or phenolic derivatives in the vacuoplasm.

Iron II sulphate, used as a general test for tannins in the light microscope (Johansen, 1940), is found to provide sufficient electron density in its reaction product to be useful in the electron microscope also. In many systems it is unsuitable due to the problems of penetration into the tissue, but in Dionaea, with a very short diffusion pathway, it proved satisfactory.

Fig. 4.22 shows a light micrograph of a gland stained for tannin. Although there is some reaction product present throughout the cells it is particularly heavily concentrated in the vacuoles. Fig. 4.23 shows an electron micrograph of material subjected to this treatment, in which the vacuoles are lined with a dense deposit of reaction product, which is absent in the controls (Fig. 4.24). The very even depth of penetration into the vacuole implies that penetration was limited by the deposition of reaction product, as often appears to be the case in glutaraldehyde - osmium tetroxide fixed material.

5) Autoradiography

Table 4.1 summarizes the distribution of silver grains observed in the secretory cells of the gland, both in the mature state and an immature state about three days before maturity. The calculated total grains are the numbers that would be expected if that number of grains were randomly distributed across the gland section, based on the known structure of the cells as given in Table 3.1.

The χ^2-tests show that in both the inner and outer secretory cells the distribution differs significantly from that predicted by calculation. Furthermore, there are significant differences between the mature and immature states of the cells. Because the overall grain density is very low (Fig. 4.25) it was not possible to record the distribution in a way that allowed a more rigorous statistical analysis to be applied. There are, however, a number of factors which clearly make major contributions to the overall difference between the observed and calculated distributions.

The large vacuolar compartment (V_l) of the mature outer cells contains considerably fewer grains than predicted, while the smaller vacuoles (V_s) hold nearly four times more (Figs. 4.26 : 4.27) indicating a considerable concentration of radiolabel in this latter compartment. This is not the case in the mature inner cells where there is only a slightly larger number in these compartments than expected. This is probably a reflection of the much greater volume percent of the small vacuoles in the inner cells, where they almost equal the large vacuole in size (Table 3.1).

Both cell layers, however, show a very large concentration of radiolabel in the labyrinthine wall (Fig. 4.28). The primary wall of the outer cells shows the same grain-percent content as the immature cells, which lack labyrinthine wall. Yet, although in the mature unstimulated state this wall only occupies 2% of the cross-sectional area of the cell, it contains 9.5% of the grains.

The nucleus of mature inner and outer secretory cells has a rather lower number of grains than predicted although the percentages of grains in the immature cells are as would be predicted if the nucleus occupies an equivalent volume in the immature cells as in the mature cells. Possibly there is a drop in nuclear protein synthesis during maturation, as it seems unlikely that the nucleus will decrease in volume by 50% over this period.

Mitochondria (Fig. 4.29) have much the expected number of grains, while the density over plastids is rather lower. Even allowing for the area occupied by endoplasmic reticulum (of mean width 61 nm : 100 random measurements) the grains over the ground-plasm are very low, particularly in the inner secretory cells, whereas the number over the endoplasmic reticulum greatly exceeds the predicted value, as might be expected. Furthermore, there is a very high concentration over the smooth endoplasmic reticulum (Fig. 4.26 : 4.30 : 4.31) which is much more prevalent than rough endoplasmic reticulum at this stage, but in the outer, and probably the inner, secretory cells the rough endoplasmic reticulum also exceeds the predicted level (Fig. 4.32).

Although the dictyosomes and dictyosomal vesicles do

have a few more grains than predicted (Fig. 4.29) the ratio is very much less than in the endoplasmic reticulum, implying that the functions of these two organelles are not closely linked.

Multivesicular bodies, virtually absent in unstimulated mature gland cells, account for about 3% of the grains in the immature tissue.

6) Density Labelling

The possibility of showing protein synthesis by density labelling arose following the preliminary experiments in which radiolabel became incorporated into the protein fraction (see 4:II:7). Plants were stimulated to secrete in the presence of ^2H_2O and secretion collected daily.

The results of two of these experiments are shown in Figs. 4.33 and 4.34, and the details are given in Table 4.2. These figures show, respectively, the buoyant densities of acid phosphatase in secretion collected early (Fig. 4.33) and late (Fig. 4.34) in the secretory cycle from traps stimulated with and without ^2H_2O in the stimulating solution. In neither case is there any difference between the buoyant density of the control and that of the treated sample. Neither is there any difference in buoyant density due to the different concentrations of ^2H_2O used. There is, however, a large inhibition of the acid phosphatase activity in the presence of ^2H_2O which is related to the concentration of ^2H_2O present (Table 4.2).

7) Radiolabelling

Secretion was collected daily from plants being stimulated with 4%(w/v) bactopeptone solution containing L-[U-^{14}C]-leucine. Aliquots of this were applied to a Sephadex G-25 column with and without pre-treatment by precipitation in 80%(v/v) ethanol. The elution profiles obtained for acid phosphatase activity, radioactivity and extinction at 280 nm are shown in Fig. 4.35. A comparison with Fig. 2.1 shows that the initial peak should contain all the protein and that the smaller second peak in the radioactivity profile is due to unassimilated radiolabel. There is no acid phosphatase activity associated with the latter peak in the untreated secretion and only a small amount of activity remains under the main peak (26.6%) after ethanolic precipitation. Most of the radioactivity (72.3%) under the main peak is removed by treatment with 80%(v/v) ethanol, whereas this has a negligible effect on the peak due to unassimilated radiolabel.

To check that this incorporation was not due to microbial contamination, the experiment was performed with benzylpenicillin and benlate fungicide included in the stimulating solution. Fig. 4.36 shows that association of radiolabel with the protein, acid protease and acid phosphatase peaks still occurs in the presence of these inhibitors of microbial growth. The much larger size of the peak of unreacted radiolabel is due to the use of L-[4,5-^{3}H]-leucine in this experiment rather than L-[U-^{14}C]-leucine, of which a much higher proportion became incorporated, as shown by comparing

this profile with that of Fig. 4.35.

When the eluant from a protein peak of Sephadex G-25 chromatography is dialysed for 48 h against buffer, over 97% of the radioactivity remains with the non-diffusible material (Table 4.3). This indicates that the association of radiolabel with the protein peak is not due to non-specific binding of unreacted L-[U-^{14}C]-leucine to the secreted proteins. Similarly, when this preparation is treated by passing it through a cation-exchange bed of Amberlite IR-120, only 12.5% of the acid phosphatase activity and 15.4% of the radiolabel remains bound to the column, the rest remaining in the eluant which contains about 62% of the protein. Free L-leucine, under the conditions used, should remain bound to the ion-exchange resin. Anion-exchange resin proved unsatisfactory as the enzyme activity is lost overnight (see 2:IV:5).

It is at this stage that the density labelling experiments reported in the previous section were attempted and, subsequent to these, it was decided to try and show an association of radiolabel with the enzymatic activities of the secretion by isoelectric focussing. The results of this are shown in Fig. 4.37. One of the major bands of radioactivity corresponds exactly with the acid phosphatase activity of the secretion (pI 3.9). The other presumably represents one of the major proteases, as may the shoulder at pI 4.0, part of the peak corresponding to the acid phosphatase, and the small peak at pI 3.7. It proved impossible to measure the protease activity in the gels by the ninhydrin method as one of the components of the gel,

probably the ampholine, reacted strongly with the ninhydrin reagent. The radiolabel at around pI 6.0 is presumably due to unreacted L-[U-^{14}C]-leucine (pI 6.036 : Smith et al, 1937) and material which did not run into the gel.

In addition, the two main peaks of radiolabel and the shoulder at pI 4.0 correspond very well to the protein bands obtained by staining the gel with Coomassie brilliant blue R.

When traps are stimulated to secrete in the presence of cycloheximide (Table 4.4), the specific radioactivity of the secretion decreases, and the decrease is related to the concentration of cycloheximide present. The specific activities of acid phosphatase and acid protease also both show a strongly dose-correlated decrease for concentrations of cycloheximide greater than 10^{-7}M but, surprisingly, the activity at 10^{-7}M- is greater than at 10^{-8}M- cycloheximide. The concentration of protein secreted, and the volume of secretion are, however, unaffected.

To confirm that the incorporation of radiolabel into protein was taking place within the leaves of traps fed with L-[4,5-^{3}H]-leucine and was not due entirely to microbial activity in the trap, tissue was excised from the plant, thoroughly washed to remove surface contamination, and homogenized as described in Table 4.5. It was found that radiolabel was present within the leaves and that over 90% was incorporated into the TCA-precipitable fraction.

III DISCUSSION

1) Storage of the Secreted Proteins

One of the major problems concerning the activity of the Dionaea secretory cells that Schwab et al (1969) felt they were unable to solve was where the hydrolytic enzymes are stored. They observed the intensely osmiophilic nature of some of the vacuoles and, on the basis of the staining of wax-embedded material with Sudan III and ninhydrin, interpreted this as due to the vacuoles being a lipo-protein complex. On the basis of this, and the lack of any other suitable organelle to fulfil this role, they proposed that the vacuole was the site of storage of acid hydrolase activity. The data presented within this chapter confirm the vacuoles as sites of hydrolase activity, but show that it is probable that not all of the vacuolar compartments are sites of storage for the enzymes found in the secretion.

Acid phosphatase is present in both the large and small vacuoles of unstimulated secretory cells while the presence of protein is confirmed by the staining of tissue with mercury II - bromophenol blue and by the loss of contrast in the vacuoles when thin sections are treated with pronase. The proportion of fixed L-[4,5-^{3}H]-leucine within the large vacuoles is very much less than would be expected if the label was evenly distributed, let alone if there was a concentration of radiolabelled protein within the vacuoplasm. Therefore it is possible that this vacuole is not as important a site for the storage of acid hydrolase activity as was initially believed. The small vacuoles, however, are

associated with a much higher proportion of the silver grains than predicted, and might therefore be much more important than the large vacuoles as a site of storage for the secretory proteins. The redistribution of silver grains following stimulation, which will be examined in Chapter 5, lends support to this premise. It was demonstrated, by the methods used in 4:II:7, that the secretion obtained from these traps did contain radiolabelled proteins.

In contrast to the results of Schwab et al (1969), however, the vacuole does not appear to contain much lipid. In the light microscope it shows little affinity for Sudan IV (Fig. 3.16) while the digestion of thin sections with lipase has negligible effect on the contrast of the vacuoles of the secretory cells. Instead, it is demonstrated that much of the osmiophilic matter may be secondary compounds. The vacuole stains green with iron III chloride in the light microscope and takes up considerable iron from iron II sulphate, as seen in the electron microscope. It seems extremely likely that these staining properties are due to the bright red anthocyanin present in the secretory cells, which is clearly seen to be in the vacuoles when fresh material is viewed under a stereoscopic light microscope. This has been shown to be cyanidin-3-glucoside (Di Gregorio and Di Palma, 1966). Similar staining properties with osmium tetroxide are found in Kalanchöe blossfeldiana where the vacuoles are also known to contain large quantities of anthocyanin (Gifford and Stewart, 1967). Furthermore, in Nepenthes and Drosera the glands are often brightly coloured red, presumably due to anthocyanins, and stain

intensely with osmium (but for Drosera see 3:IV:3) whereas in Utricularia the glands are not coloured and do not stain intensely with osmium. In Pinguicula the coloration and staining properties are both variable.

It thus appears that the osmiophilic vacuoles represent an anthocyanin-protein, rather than lipo-protein, complex in which some of the protein is acid phosphatase.

Within the walls of the secretory cells, acid phosphatase is detected only in the primary wall region, being conspicuously absent from the labyrinthine part of the wall. Such a marked segregation seems highly irregular and is possibly an artefact due to the acid phosphatase in the labyrinthine wall being inactivated either by a deliberate inactivation mechanism or by the pH of the wall in this region being elevated. Scala et al (1969) have shown the acid phosphatase in the secretion to be virtually completely inactive above pH 7.0. The improvement in the staining of the labyrinthine wall with toluidine blue obtained by Gunning and Pate (1974) when sections were pre-treated with 0.01M-hydrochloric acid indicates that the material of which these walls are composed may have an alkaline pH and this may be sufficient to prevent any reaction. Alternatively the acid phosphatase within the labyrinthine wall may be very loosely held and washed out into the primary wall during fixation and incubation. The lack of lead II phosphate deposits within this part of the wall is not due to poor penetration of the substrate as otherwise no activity would be detected within the cells.

The autoradiography indicates that a high level of protein is present in the labyrinthine walls, several times more concentrated than in the primary walls, although this part of the cell wall shows very little staining with mercury II - bromophenol blue. Under ordinary glutaraldehyde - osmium tetroxide staining, both parts of the cell wall show insufficient electron density to determine whether or not the pronase has removed material from this region.

It therefore appears that the labyrinthine wall contains a good deal of protein which is very low in thiol content, and thus is less reactive with mercury II - bromophenol blue (Pearce, 1960), and is either not acid phosphatase or any acid phosphatase present is in an inactivated form.

Previously it has been found (Robins, 1975) that the secreted proteins contain negligible amounts of cystine and only very small amounts of methionine (Table 4.6). Hence, while this implies that the strong reaction in the vacuole with mercury II - bromophenol blue may not involve secretory proteins, it does indicate that the labyrinthine walls could contain secretory proteins which are not being detected by this stain.

Acid phosphatase is commonly detected in plant tissue at the level of the electron microscope. It has been found in almost all cellular compartments, although it is particularly common in the vacuoles and/or cell walls. The vacuoles of polysaccharide-secreting systems, notably root-tip cells (Hall and Davie, 1971; Poux, 1970; Sexton *et al*, 1971) and the squamule cells of *Elodea* (Rougier, 1972) all stain strongly for acid phosphatase. In these systems the

dictyosomes, vesicles derived from the dictyosomes, and the cell walls also show acid phosphatase activity.

As yet no high resolution localization of acid phosphatase has been performed in one of the carnivorous plant glands known to secrete predominantly polysaccharides. In the light microscope Heslop-Harrison (1975) has found this activity within the stalked glands of Drosera and Drosophyllum. She shows that, in the latter, it is localized within the cell walls of the secretory cells, no activity being found in the endodermis and tracheid mass. In Pinguicula the stalked glands, which secrete polysaccharides and protein, show no acid phosphatase activity within the cells but a strong reaction within the cell walls, whereas the sessile glands, secreting primarily protein, show the same intense reaction in the cell walls but some acid phosphatase also localized within the vacuoles of the gland-head cells (Heslop-Harrison and Knox, 1971). Acid phosphatase has been localized in Utricularia using similar techniques to those used here. Vintéjoux (1974a, b) finds a strong reaction in the dictyosomes, vesicles and labyrinthine walls of the basal cells of the quadrifid glands, but not in the vacuoles. The cap cells of the gland also show enzyme activity in the labyrinthine walls, but the vesicles and vacuoles are devoid of reaction product. The mitochondria, endoplasmic reticulum and plasmalemma of the cap cells also show lead II phosphate deposits, and it is therefore possible that some of this activity is due to other phosphates - such as membrane bound ATPases. No examination was made of ATPase activity to check for this.

Thus it appears that within the other genera of carnivorous plants that have been investigated acid phosphatase can be localized, as with Dionaea, in both the walls and vacuoles of the secretory cells. Several other hydrolase activities have also been detected within the glands of carnivorous plants at the level of the light microscope, and these are summarized in Table 4.7. Apart from the detection of E-600 resistant esterase activity in the vacuoles of the stalked glands of Drosera (Palczewska, 1966), Pinguicula is the only genus in which the subcellular distribution of some of these enzymes has been analysed (Heslop-Harrison and Knox, 1971). Esterase follows a very similar distribution to acid phosphatase, while ribonuclease is principally in the labyrinthine walls but also detected in the nucleus and external walls of the sessile gland cells. In Genlisea Heslop-Harrison (1976) has shown the labyrinthine walls to contain high concentrations of protein compared to the rest of the cell wall and the cell cytoplasm.

It thus appears that several genera, if not all, store the secretory proteins within the labyrinthine and/or primary cell walls of the secretory cells and that in addition some genera have an intracellular store in a vacuolar compartment. It is no surprise to find these hydrolytic enzymes being stored within the vacuoles of the secretory cells. The vacuole is now considered an important component of the lysosomal compartment of the plant cell (Matile, 1974) and therefore might be expected to contain acid phosphatase, a standard marker for lysosomes. What is difficult to ascertain is whether these vacuolar enzymes are

stored ready for secretion or whether they have a primarily intracellular function. This will be considered further in Chapter 5.

The smooth endoplasmic reticulum in both the inner and outer mature secretory cells has an extremely high concentration of radiolabel - very much greater than predicted and also very much greater than in the rough endoplasmic reticulum (Table 4.1). This suggests that these may be an important site for the storage of secretion in a form from which it can be rapidly released from the cells (see Chapter 5). The profiles of endoplasmic reticulum do not stain intensely with mercury II - bromophenol blue (Fig. 4.14) but some activity of acid phosphatase is detected within them (Fig. 4.2). It cannot, unfortunately, be ascertained whether this is smooth or rough endoplasmic reticulum in these preparations.

The data presented in Table 4.1 suggest that, as the secretory cells mature, protein is made on the rough endoplasmic reticulum and migrates, via profiles of smooth endoplasmic reticulum, to the small vacuoles. This will be discussed further in 4:III:2, but the conclusion is based on a comparison of the proportions of silver grains within these compartments in the mature and immature tissues.

Although the ontogeny of the vacuolar compartments has not been examined in Dionaea there is evidence from other sources that the endoplasmic reticulum makes a major contribution to the growth of the vacuole in a number of protein-storing systems. In the root meristem of Cucurbita (Coulomb and Coulon, 1971), the nucellus of Euphorbia (Gori et al,

1971) and developing seeds of Vicia (Bailey et al, 1970) and Zea (Khoo and Wolf, 1970) the protein bodies are derived directly from the endoplasmic reticulum. Bailey et al (1970) demonstrate this by pulse-chase autoradiography. With many other species, however, the development of a protein store involves an intermediate vacuolar stage. This is seen, for example, in the production of the wound-healing protein bodies of Bryopsis (Burr and West, 1971; Burr and Evert, 1972) as well as in the development of reserve protein grains in the seeds of Phaseolus (Öpik, 1968b), Sinapis (Rest and Vaughan, 1972) and Capsella (Schultz and Jensen, 1968).

No connections have been seen between either smooth or rough endoplasmic reticulum and vacuoles in either stimulated or unstimulated secretory cells. There is, however, overwhelming evidence, reviewed by Matile (1974), that cell vacuoles are derived from endoplasmic reticulum either by local dilations of the cisternae or by vesiculation of parts of the membrane system. Therefore, although a migration pathway for protein from the endoplasmic reticulum to the small vacuoles remains conjectural in Dionaea, there is plenty of evidence to support the possibility that it could exist. The way in which profiles of endoplasmic reticulum are frequently found closely juxtaposed to vacuoles, particularly in stimulated tissue, strengthens the idea of a close functional correlation between these two bodies.

The distribution of ATPase activity within the cell is much as expected with considerable activity along the plasmalemma, some along the tonoplast and some in the mitochondria

and ground-plasm (Hall, 1971). The activity within the labyrinthine wall deserves comment. In a recent study of ATPase localization in the phloem of Pisum, Bentwood and Cronshaw (1978) show ATPase in the plasmalemma surrounding labyrinthine invaginations but not within the wall itself. The labyrinthine wall in Pisum, however, is much less convoluted than in Dionaea. In the latter some activity is apparently located within the labyrinthine walls (Figs. 4.6 : 4.7) but much of this may arise from glancing sections of cytoplasmic invaginations. Unfortunately in these preparations the plasmalemma is often not well stained and thus not clearly defined. The deposits of lead II phosphate in Fig. 4.7 are concentrated around regions of dark-staining material which has the appearance of cytoplasm intercollated with the labyrinthine wall protruberances (compare with Figs. 3.35 : 3.36). Some of the deposits, which show no relationship to any such structure, may represent soluble ATPase activity within the labyrinthine wall as Scala et al (1969) detected ATPase activity within the secretion. Maier and Maier (1972) also found deposits of lead II phosphate from ATPase activity in the transfer cells of a moss haustorium and considered this to be associated with the fibrillar matter which forms the core of these walls. The areas of high ATPase activity within Fig. 4.7 are not core material and no such association was found in Dionaea. With their tissue, incubated for 180 min, the lead II phosphate deposits were unevenly distributed across the plasmalemma, with more deposition towards the cell interior. In Fig. 4.11, in which the cell has been lysed and the tissue

incubated only half as long the deposits are entirely to the inward-directed face of the plasmalemma. Because the cell is lysed this distribution cannot have been caused by unequal concentrations of the substrate at the two sides of the membrane. Therefore such an image presents evidence that the ATPase localized within the plasmalemma releases the inorganic phosphate produced by its activity at the inner face of the membrane.

2) Synthesis of the Secreted Proteins

Since it has already been established that some of the protein released from the glands on stimulation comes from a site of storage, it is to be expected that considerable protein synthesis is occurring during the development of the trap. It was unfortunately not possible to follow the distribution of radiolabel and the ultrastructural changes within the glands during development due to a combination of problems with the radiolabelling of the plants and the fixation of this material. Table 4.1, however, shows that just a few days before maturation (the complete development of a leaf taking about 14 days) there is a considerably higher percentage of the silver grains present over the rough endoplasmic reticulum than there is once the leaf has matured. This indicates that there is a higher rate of synthesis of protein during the development of the trap than after maturation. The proportion of silver grains over the smooth endoplasmic reticulum is also very high in the immature traps but does not decrease at maturation.

Furthermore, the proportion of silver grains over the small vacuoles is very much less in the immature than in the mature traps. Thus it can be proposed that during this phase protein destined for secretion is being synthesized on the rough endoplasmic reticulum which at a certain point loses its ribosomes, presumably in response to a high concentration of protein in the cisternae, and becomes smooth. These smooth profiles of endoplasmic reticulum may then be involved in the production of the small vacuoles, which thus form an important intracellular store of protein ready to be secreted. Some of the reticulum membranes, both rough and smooth, however, apparently fuse to the plasmalemma and secrete their products directly into the extracellular space, forming another important store in the labyrinthine and primary cell walls. The effect of stimulation on these stores is considered in Chapter 5.

Previously it has been assumed that carnivorous plants store the protein to be secreted and secrete it in response to stimulation. In Dionaea the stores are then re-charged ready for further activity. Autoradiographic evidence is presented in Chapter 5 which demonstrates for the first time that these stores are being re-charged. Furthermore, the evidence presented here is the first demonstration in carnivorous plants that protein destined for export is synthesized during the secretory phase of activity and that at least some of this is used during that same period of secretion.

Fig. 4.35 demonstrates that when plants are fed

radiolabelled L-leucine through the leaves then a considerable proportion of the activity present in the secretion 24 h later is within the proteinaceous fraction. Fig. 4.36 shows that this incorporation is not due to microbial contamination. Furthermore the radiolabel is not non-specifically bound to the secreted protein as the two cannot be separated either by dialysis or by ion-exchange chromatography (Table 4.3). This incorporation is found to be significantly depressed (χ^2-test: $p<0.01$) by cycloheximide (Table 4.4), although the drug does not significantly affect the concentration of protein in the secretion (χ^2-test: NS). There is, however, a large dose-dependent decrease in the levels of activity of both acid protease and acid phosphatase in the secretion from plants treated with cycloheximide. Thus the drug appears to inhibit the synthesis of these enzymes and simultaneously disrupt the selection of proteins for secretion. This latter effect is also observed with colchicine and cytochalasin B and will be considered further in Chapter 5.

Radiolabelling is normally not considered a satisfactory technique for demonstrating the de novo synthesis of an enzyme due to the technical problems of convincingly demonstrating that the radiolabel is incorporated into the enzyme under consideration. In the Dionaea system, however, the plant effectively purifies the proteins required by secreting them into the extracellular fluid. Hence the demonstration, under the conditions of study, of radiolabel incorporated into the secreted protein should itself be sufficient evidence that de novo synthesis of proteins, which are then being secreted, does occur. Further confirmation of this

was sought, however.

The technique of density labelling avoids the purification problems of radiolabelling and, under suitable conditions, readily provides the necessary demonstration of de novo synthesis. This method, however, does not detect any de novo synthesis of acid phosphatase during secretion, as shown by Figs. 4.33 and 4.34. As it is unlikely that the whole population of acid phosphatase would contain ^{2}H-label, the shapes of these curves were examined using normal-probability distribution plots. If a portion of the population contains ^{2}H-label then this will be concentrated in the part of the peak to the denser side of the mean (or, as plotted here, higher values of relative buoyant density) and thus cause a skew distribution in the density-labelled sample. Figs. 4.33b and 4.34b show that while the peak of acid phosphatase activity in the former is skew, the degree of skewness is the same in both treated and control samples, while in the latter the peak is not skewed at all. This indicates that the populations of acid phosphatase molecules contained no density label.

Hence from these experiments it could be concluded either that, in contrast to the radiolabelling evidence, de novo protein synthesis does not occur or that this technique is not suitable to detect synthesis in this system. $^{2}H_{2}O$ is an established inhibitor of protein synthesis when exhibited at high concentrations. Lamb et al (in press) have recently shown severe inhibition of the light-induced de novo synthesis of phenylalanine ammonia-lyase in discs of potato tuber parenchyme when final concentrations of $^{2}H_{2}O$

in excess of 50%(v/v) are present. Because a Dionaea lobe does not present a 'closed' system, ^2H_2O was exhibited at high external concentrations (50 and 80%(v/v)) in order to try and obtain the approximately optimal internal concentration of 40%(v/v) (Lamb et al, in press). Table 4.2 shows that with both of the concentrations of ^2H_2O used the activity of acid phosphatase in the treated samples is very much lower than in their controls and that the degree of inhibition is related to the external concentration of ^2H_2O present. Therefore it appears that the ^2H_2O is in fact severely inhibiting protein synthesis instead of demonstrating it. Furthermore, this technique is most suitable when applied to systems in which the background level of enzyme is very low in comparison with the level following stimulation. This is clearly not the case here and so the application of this technique to the system under study is probably invalid.

The association of radiolabel with one of the secreted enzyme activities - the acid phosphatase - was, however, demonstrated by isoelectric focussing (Fig. 4.37). Secretion collected from plants fed L-[U-^{14}C]-leucine shows co-banding of acid phosphatase activity with one of the major bands of radioactivity and one of the bands of protein.

It seems probable that the other major peak of radioactivity (pI 4.0) represents one or more of the proteases present in the secretion (see Chapter 6), but this needs to be examined using an alternative method for estimating the protease activity. It is highly unlikely that these peaks of radioactivity are due to unreacted L-[U-^{14}C]-leucine

non-specifically bound to the proteins as the pH gradient ensures that any free L-leucine migrates to its own isoelectric point at pI 6.036 (Smith et al, 1937).

Ultrastructural support for the concept of protein synthesis occurring in the secretory cells following stimulation is provided by the observed changes in the form of the endoplasmic reticulum and ribosomes within these cells. As reported in Chapter 3, the resting cells contain numerous profiles of endoplasmic reticulum, a large proportion of which is without ribosomes, while there are a great many ribosomes free in the cytoplasm. After stimulation, the majority of ribosomes are found bound to the endoplasmic reticulum, mostly as polysomes.

Briarty et al (1969) observed a similar increase in rough endoplasmic reticulum when developing seeds of Vicia started making protein granules while Jensen (1965) and Gori et al (1971) both found large numbers of polysomes on the endoplasmic reticulum making the protein bodies in the nucelli of cotton and Euphorbia respectively. Polysomes were also found on the surfaces of the distended cisternae of endoplasmic reticulum in Raphanus root-hairs, where massive protein production is apparently taking place (Bonnett and Newcomb, 1965).

Warner et al (1962) have shown by sucrose gradient ultracentrifugation that polysomes are responsible for synthesizing haemoglobin in reticulocytes, while free ribosomes are not. Marks et al (1963) further showed that the level of production of haemoglobin in this system greatly increases when polysomes first appear in large numbers and

steadily decreases in parallel with decreasing numbers of polysomes. From this and other evidence it has become generally accepted that polysomes are associated with active protein synthesis. There is also considerable evidence that proteins destined for secretion are synthesized by polysomes bound to endoplasmic reticulum while those made on cytoplasmic polysomes are not secreted (Campbell, 1970; Scheele et al, 1978).

Altogether the observed changes in the ultrastructure of the secretory cells are totally compatible with a stimulus-induced switch from a tissue undergoing only low rates of protein synthesis to one in which much higher rates of protein synthesis occur. Micrographs in which rough endoplasmic reticulum is seen fused directly to the plasmalemma lend support to the thesis that some of this protein is secreted directly while images such as Figs. 3.65 - 3.67 support the autoradiographic evidence that some of the protein to be secreted passes into small vacuoles within the cells (Tables 4.1 and 5.2). The passage of this secretion out of the cells is the subject of the following chapter.

Table 4.1: The Distribution of Silver Grains within the Gland Cells Before Stimulation

		Silver Grain Distribution	Cytoplasmic Compartment															
			1°CW	LW	V_l	V_s	MVB	N	M	LD	P	D	DV	sER	rER	TER	GP	Total
Outer Secretory Cell	Mature	Observed % grains present	14.4	9.5	25.5	17.1	0	1.5	3.4	0	0.8	2.3	1.9	12.9	1.9	-	8.4	-
		Observed total grains present	38	25	67	45	0	5	9	0	2	6	5	34	5	-	22	263
		Calculated total grains	32	5	109	12	<1	26	10	0	5	4	1	-	-	1	58	263
	Immature	Observed % grains present	12.9	-	32.5	7.1	2.8	6.0	3.4	0.3	0.9	0.9	1.4	15.2	4.7	-	4.8	-
		Observed total grains present	91	-	229	50	20	42	24	2	6	6	10	107	33	-	84	704
Inner Secretory Cell	Mature	Observed % grains present	8.1	9.4	20.8	18.8	0	6.7	8.7	0	0	0	2.0	16.1	0.7	-	8.7	-
		Observed total grains present	12	14	31	28	0	10	13	0	0	0	3	24	1	-	13	149
		Calculated total grains	13	5	23	24	0	19	10	<1	3	<1	<1	-	-	7	46	149
	Immature	Observed % grains present	13.1	-	28.8	9.3	3.1	10.0	3.1	0.2	1.7	0.7	0.5	13.6	4.1	-	11.9	-
		Observed total grains present	55	-	121	39	13	42	13	1	7	3	2	57	17	-	50	420

χ^2 tests:

Outer secretory cell:	mature observed vs. calculated	$\chi^2 = 63.65$	$p<0.001$
	mature observed vs. immature observed	$\chi^2 = 57.72$	$p<0.001$
Outer secretory cell:	mature observed vs. inner mature observed	$\chi^2 = 22.98$	$p<0.05$
Inner secretory cell:	mature observed vs. calculated	$\chi^2 = 25.18$	$p<0.01$
	mature observed vs. immature observed	$\chi^2 = 40.20$	$p<0.001$

Table 4.2: Data for the Secretion Applied to the Isopyknic Ultracentrifugation Gradients shown in Figures 4.33 and 4.34

	50% (v/v) ^2H_2O		80% (v/v) ^2H_2O	
	Control	Treatment	Control	Treatment
Concentration of protein (mg ml^{-1})	0.43	0.56	0.98	1.04
Specific activity of acid phosphatase (nkat(mg protein)$^{-1}$)	4.11	1.04	1.89	0.35
Percentage inhibition due to the presence of ^2H_2O	-	74.7	-	81.6
Number of days after stimulation	1 - 4	1 - 4	5 & 6	6 - 8
Number of traps used	10	10	8	7

Table 4.3: The Effect of Ion-exchange Extraction and Dialysis on the Relative Distributions of Protein, Enzymatic Activities, and Radioactivity

	Total Final Volume (ml)	Total Radio-activity (counts min^{-1})	Total Protein (µg)	Total Enzymatic Activities	
				Acid Phosphatase (nkat(mg $protein^{-1}$))	Protease (pkat(mg $protein^{-1}$))
Cation exchange resin IR-120:					
bound	6.0	607	367	2.7	-
free	6.0	3132	600	18.6	-
% of total free	-	83.8	62.1	87.3	-
Dialysis:					
dialysis residue	2.5	7025	1250	197.3	31.2
diffusate	600 (approx)	142	-	-	-
% of total residue	-	98.0	-	-	-

Table 4.4: The Effect of Various Concentrations of Cycloheximide on the Contents of the Secretion: Cumulative Mean after Seven Days Secretion

	Control	Concentration of Cycloheximide (M)			
		10^{-8}	10^{-7}	10^{-6}	10^{-5}
Volume ($\mu l\ mm^{-2}$)	1.42	0.99	1.26	1.11	1.48
% of control		69.8	89.2	78.2	104.4
Protein ($\mu g\ mm^{-2}$)	4.77	3.32	5.12	4.72	6.12
% of control		69.7	107.5	99.1	128.5
Protein concentration ($mg\ ml^{-1}$)	26.80	19.85	24.55	25.30	25.10
% of control		74.1	91.6	94.4	93.6
Protease activity (nkat(mg protein^{-1}))	3.71	0.78	1.88	1.24	1.06
% of control		20.9	50.5	33.5	28.6
Protease activity (pkat(mg protein^{-1})mm^{-2})	3.79	0.41	1.56	0.88	0.94
% of control		10.8	41.2	23.3	24.8
Acid phosphatase activity (nkat(mg protein^{-1}))	18.84	20.07	24.07	15.09	5.43
% of control		106.5	127.8	80.1	28.8
Acid phosphatase activity (pkat(mg protein^{-1})mm^{-2})	19.11	10.30	19.87	11.97	5.24
% of control		53.9	103.9	62.6	27.4
Specific radioactivity (counts min^{-1}(mg protein^{-1})	16156	15244	11122	14227	10141
% of control		94.4	68.8	88.1	62.8

Table 4.5: To Show the Presence of L-[4,5-^{3}H]-leucine within the Leaf Tissue

Two leaves, which had been fed for seven days with 4%(w/v) bactopeptone solution containing 2.0 μCi ml^{-1} L-[4,5-^{3}H]-leucine were excised, thoroughly washed, and frozen at -20^{o}C. After 24 h they were thawed and ground with a little acid-washed sand in 1.0 ml of cold 100 mM-sodium acetate buffer (pH5.0). Debris was removed by centrifugation and washed twice with 2.5 ml buffer. The supernatants were mixed and 3.0 ml was added to 3.0 ml 15%(w/v) TCA. After 20 min at room temperature this was centrifuged and the supernatant withdrawn (solution A). The precipitate was taken into 2.0 ml of 10%(w/v) sodium carbonate containing 0.1 M-sodium hydroxide (solution B). The debris from the initial centrifugation was suspended in 2.0 ml buffer (solution C). 1.0 ml of each of solutions A and C was decolorized with 2.0 ml 30%(w/v) hydrogen peroxide solution and 1.0 ml of this was added to 2.0 ml PPO/POPOP/Triton X 100 scintillant (2:V:4).

Solution	A	B	C
Total counts min^{-1}	450	7680	0

% counts recovered in TCA-precipitable material = 94.5%

Table 4.6: Amino Acid Composition of the Secreted Fluid

40 µl of secretion, obtained from traps stimulated with the stimulant shown, were hydrolysed overnight with 12 M-hydrochloric acid and analysed on a Locarte "Mini" automatic amino acid analyser in the Department of Biochemistry, Oxford. These results are taken from Robins (1975).

Amino Acid Present	Concentration (nmol ml^{-1})	
stimulant:	glutamine	ammonia
alanine	40.80	33.25
arginine	10.55	15.35
aspartic acid	56.00	31.75
cystine (half)	0.00	0.00
glutamic acid	- *	59.43
glycine	36.48	42.38
histidine	7.70	9.00
isoleucine	12.79	12.03
leucine	18.58	17.55
lysine	14.90	- **
methionine	0.81	3.07
phenylalanine	6.78	5.75
proline	0.00	0.00
serine	77.25	39.83
threonine	20.50	19.88
tyrosine	12.53	4.48
valine	42.80	12.75

*contaminated by added glutamine

**lysine peak obscured by ammonia

Table 4.7: Localization of Digestive Enzyme Activities within the Glands of Various Genera of Carnivorous Plants

Genus		Peroxidase	Ribonuclease	Hydrolase				Source**
				Esterase	Acid Phosphatase	Amylase	Protease	
Nepenthes		+	N/T*	+	++	N/T	N/T*	2
Drosera	(stalked)	+	N/T	++	+(CW)*	N/T	++ *	2,3
Drosophyllum	(stalked)	++	N/T	+	+(CW)	N/T	++	2
Pinguicula	(stalked)	-	+(CW)	+(CW)	+(CW)	+	+	1,2
	(sessile)	-	++(CW)	++(CW,V)	++(CW,V)	-	++	1,2
Genlisea		N/T	N/T	+	+	N/T	N/T	2
Utricularia	(quadrifid)	N/T	N/T	+	++(D,DV,LW,M,PM)	N/T	++	2
Dionaea		N/T*	N/T*	+*	++(ER,V_S,V_l,1^oCW, PM)	N/T*	N/T*	2 4

*This activity shown present in the secretion
**1 = Heslop-Harrison and Knox (1971): 2 = Heslop-Harrison (1976): 3 = Palczewska (1966): 4 = Robins (unpublished): 5 = Vintejoux (1974b)
N/T = not tested: - = no reaction: + = moderate reaction: ++ = strong reaction

Figure 4.1 Showing the presence of a dense precipitate of lead II phosphate in an outer secretory cell incubated 30 min for acid β-glycerophosphatase activity. Note heavy deposits in the vacuole and primary cell wall but conspicuous absence from cuticle and labyrinthine cell wall.

Unstimulated tissue: Spurr's resin: no poststaining. x 14,550.

Figure 4.2 Showing the presence of a dense precipitate of lead II phosphate in an inner secretory cell incubated as in Fig. 4.1. Note that, in addition to the deposits in the primary cell wall and vacuole there are some in the cytoplasm, which overlie endoplasmic reticulum (arrows). Note also the lack of reaction product in the epidermal cell wall.

Unstimulated tissue: fixed glutaraldehyde only: Spurr's resin: lead citrate poststain. x 25,300.

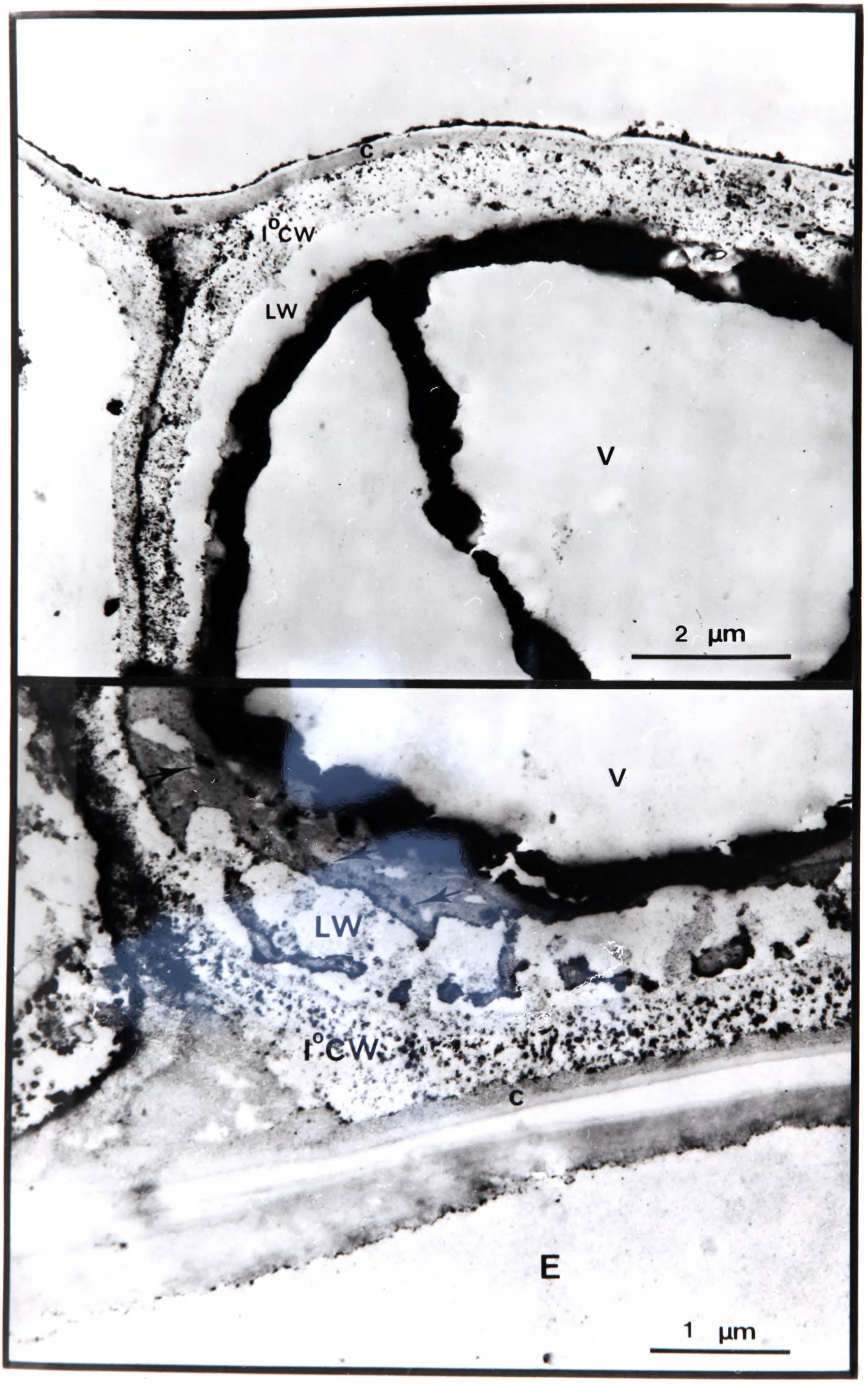

C
I°CW
LW
V
2 µm
V
LW
I°CW
C
E
1 µm

Figure 4.3 Section of an outer secretory cell from tissue incubated 30 min in medium for acid β-glycerophosphatase activity but lacking substrate. No deposits of lead II phosphate are present.

Unstimulated tissue: Spurr's resin: lead citrate poststain. x 19,970.

Figure 4.4 Parts of, and the cell wall between, an outer (upper) and an inner (lower) secretory cell, and part of the epidermal cell wall (to the right), from tissue treated as in Fig. 4.3. No deposits of lead II phosphate are present.

Unstimulated tissue: Spurr's resin: lead citrate poststain. x 23,330.

Figure 4.5 Part of the cell wall between 2 inner secretory cells incubated 30 min for acid β-glycerophosphatase activity. Note the very heavy density of lead II phosphate deposits in the middle lamella and a few deposits at the plasmalemma (arrows).

Unstimulated tissue: Spurr's resin: lead citrate poststain. x 19,580.

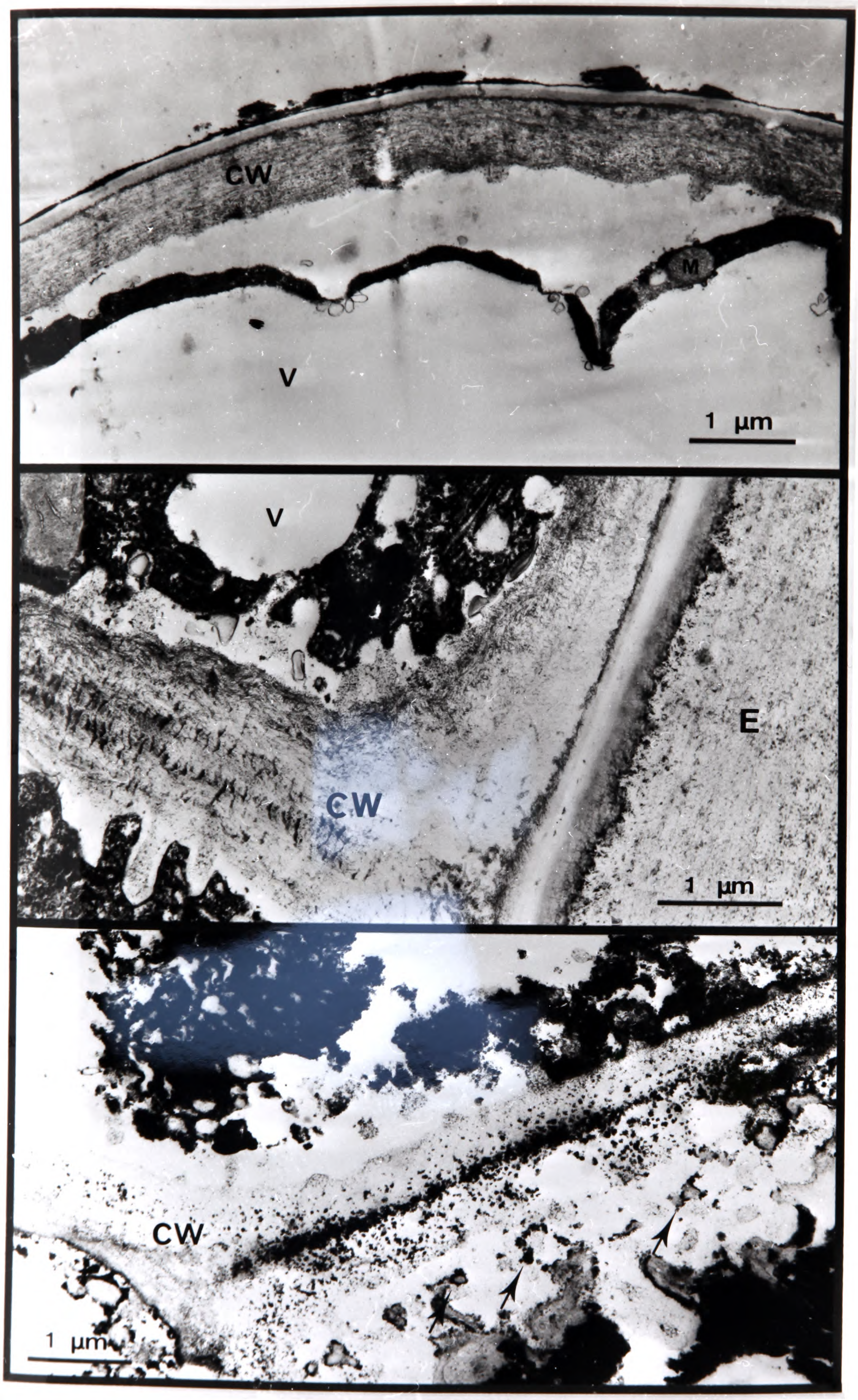

CW
M
V
1 µm
V
E
CW
1 µm
CW
1 µm

Figure 4.6 Showing part of an outer secretory cell from tissue incubated 90 min for ATPase activity. Note the lead II phosphate deposits in the labyrinthine cell wall and associated with membranes of the endoplasmic reticulum and the mitochondria.

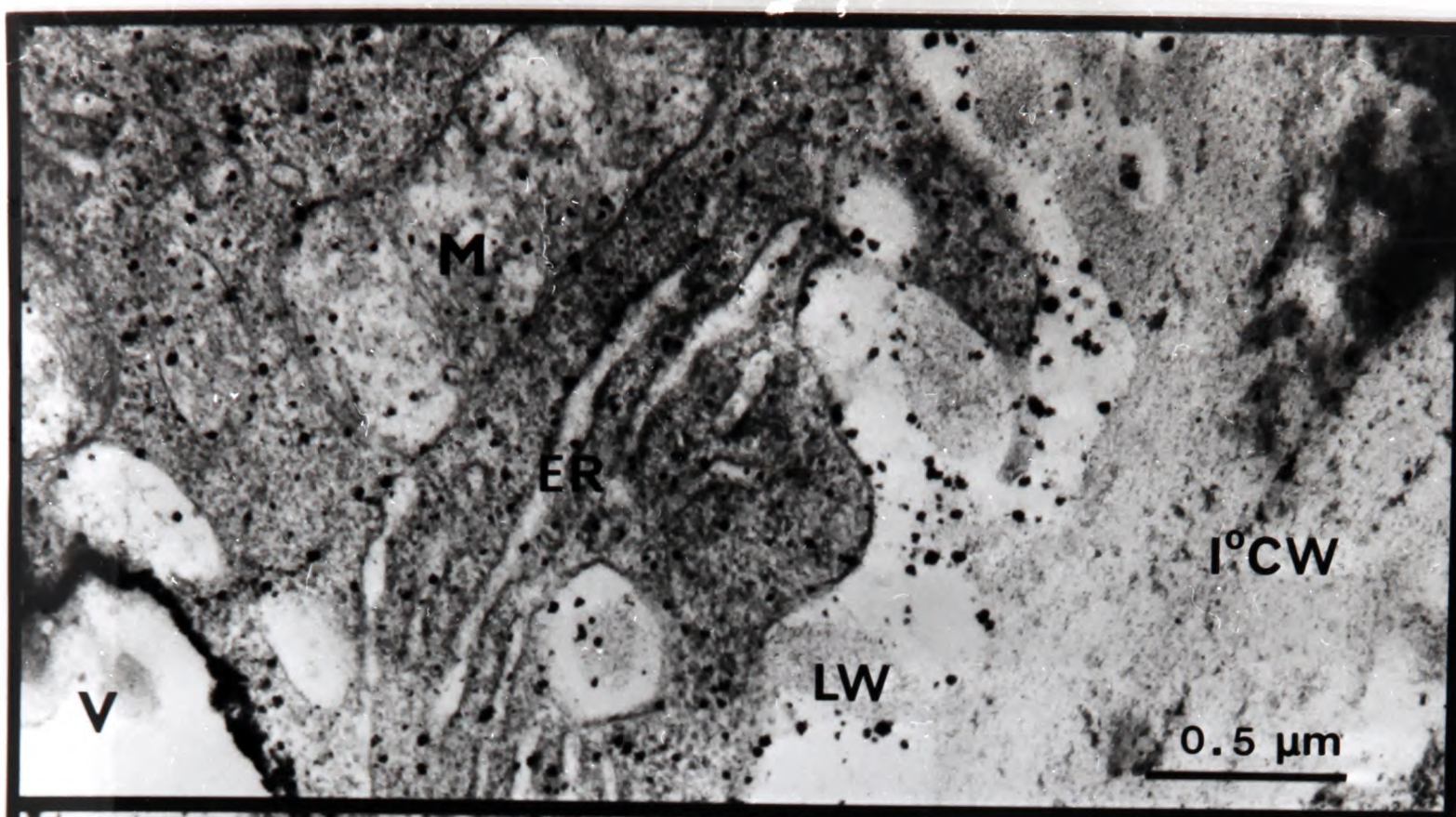
Unstimulated tissue: Spurr's resin: lead citrate poststain. x 41,860.

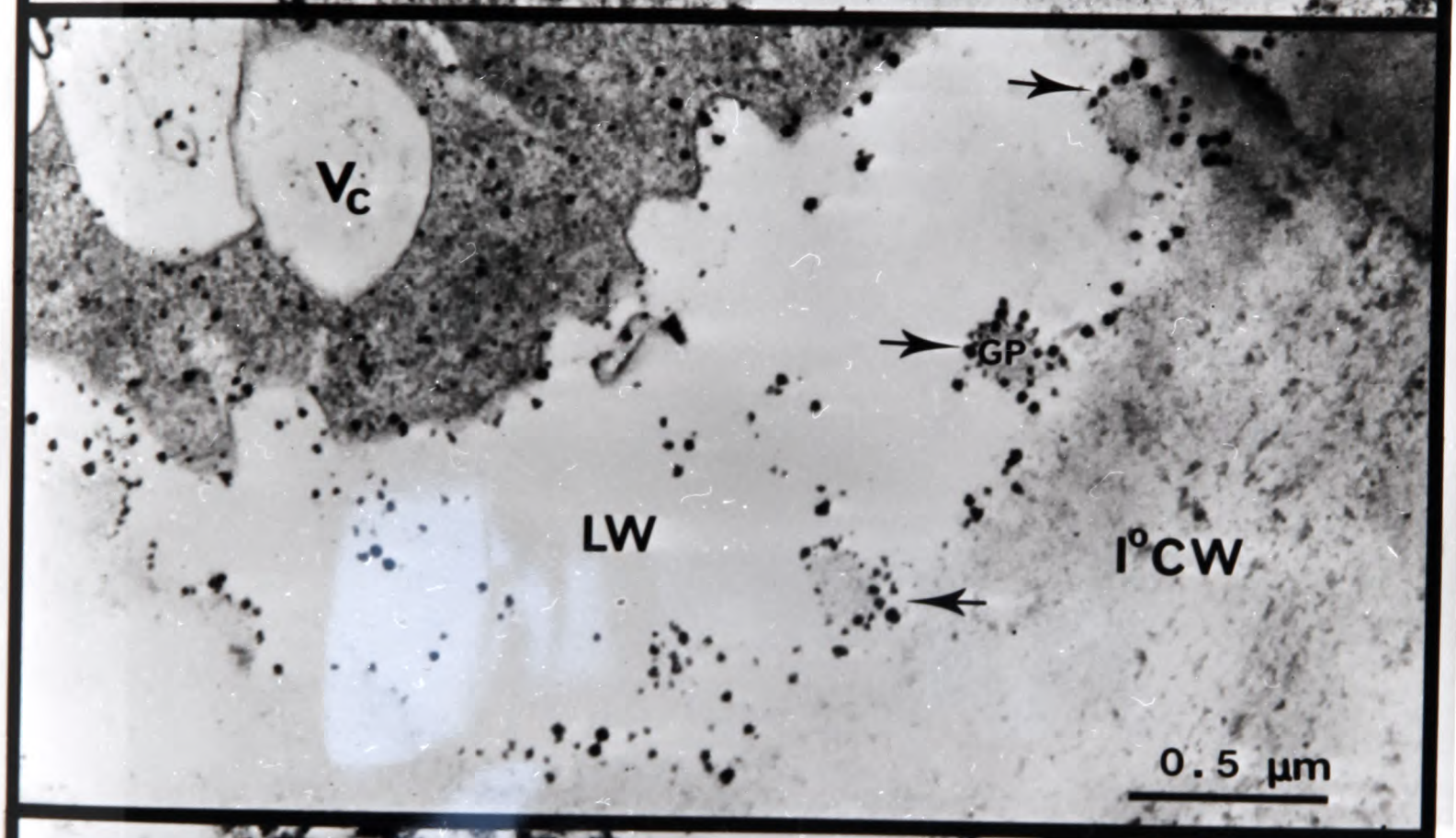
Figure 4.7 Showing the inner corner of an outer secretory cell incubated as in Fig. 4.6. Note the lead II phosphate deposits in the labyrinthine cell wall. Some of these (arrows) surround regions of cytoplasm intertwined with the labyrinthine wall but others appear free in the matrix of the wall.

Unstimulated tissue: Spurr's resin: lead citrate poststain. x 43,390.

Figure 4.8 Showing part of an outer secretory cell incubated 90 min for ATPase activity but without substrate. Note complete lack of lead II phosphate deposits but that the vacuole is heavily stained.

Unstimulated tissue: Spurr's resin: lead citrate poststain. x 42,430.


M
ER
I°CW
V
LW
0.5 µm


Vc
GP
LW
I°CW
0.5 µm

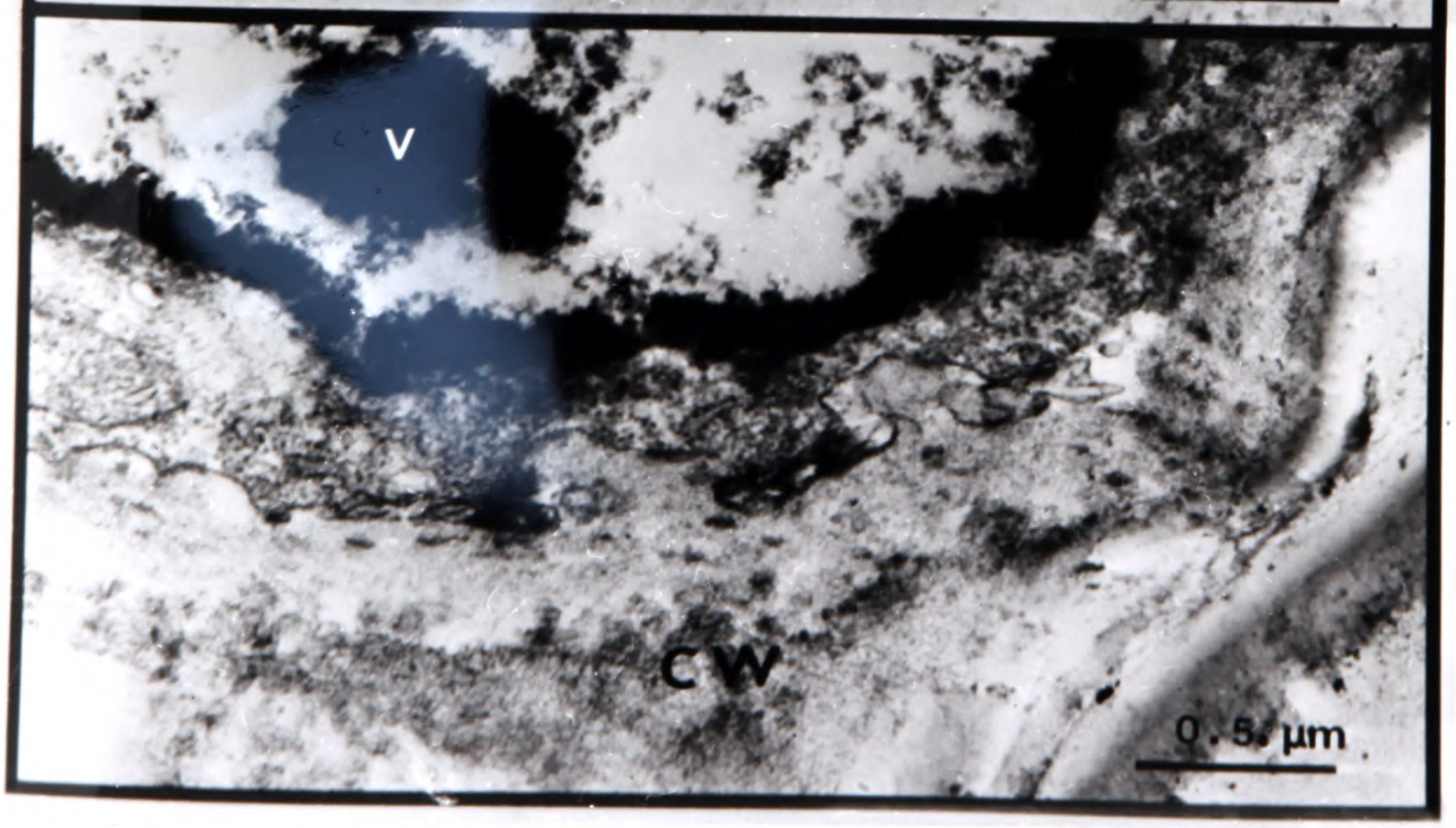

V
CW
0.5 µm

Figure 4.9 Showing part of the cytoplasm and vacuole of an outer secretory cell incubated as in Fig. 4.8. Note lack of lead II phosphate deposits in mitochondria but how the tonoplast is still stained, in contrast to Figs. 4.3 : 4.4.

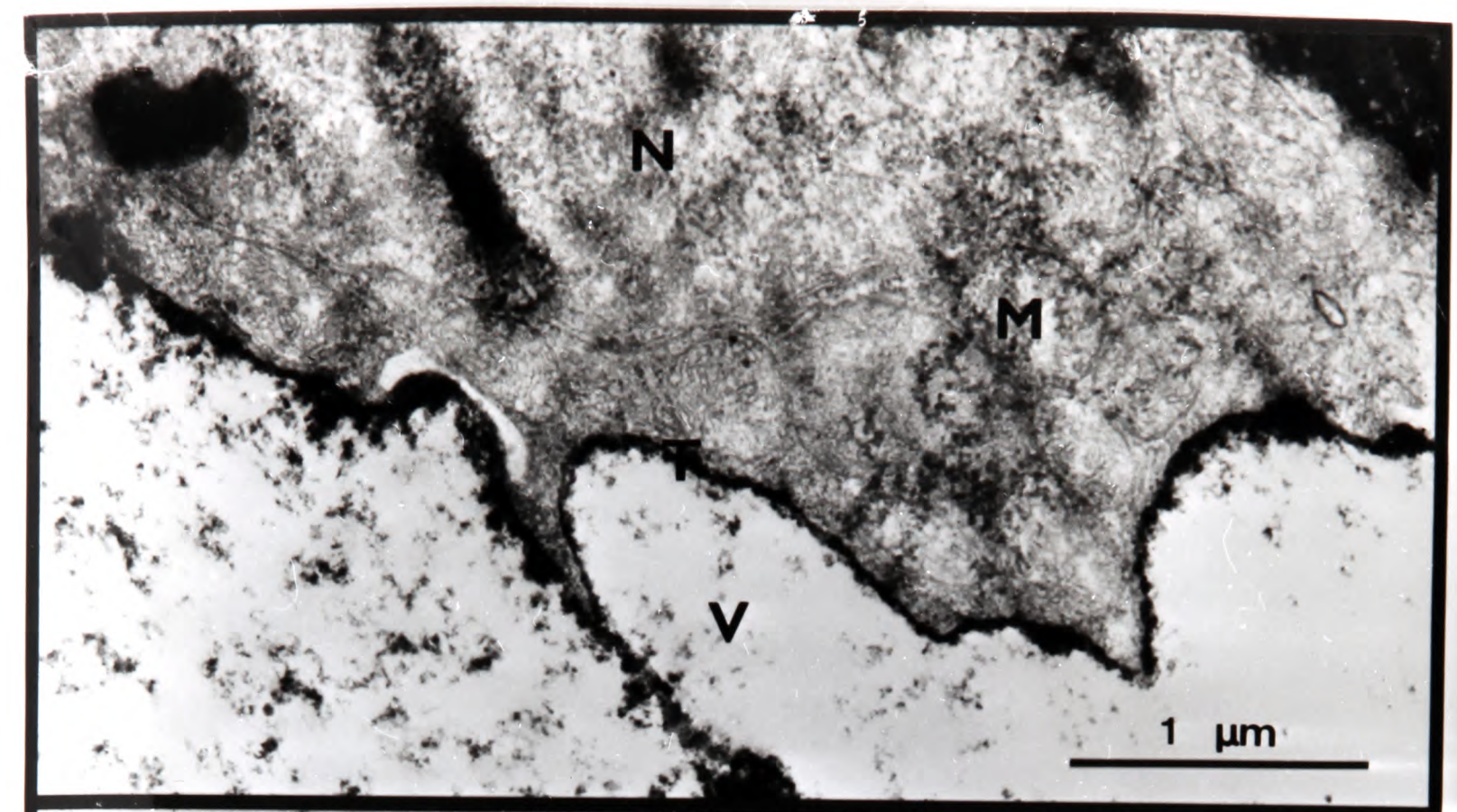
Unstimulated tissue: Spurr's resin: lead citrate poststain. x 33,130.

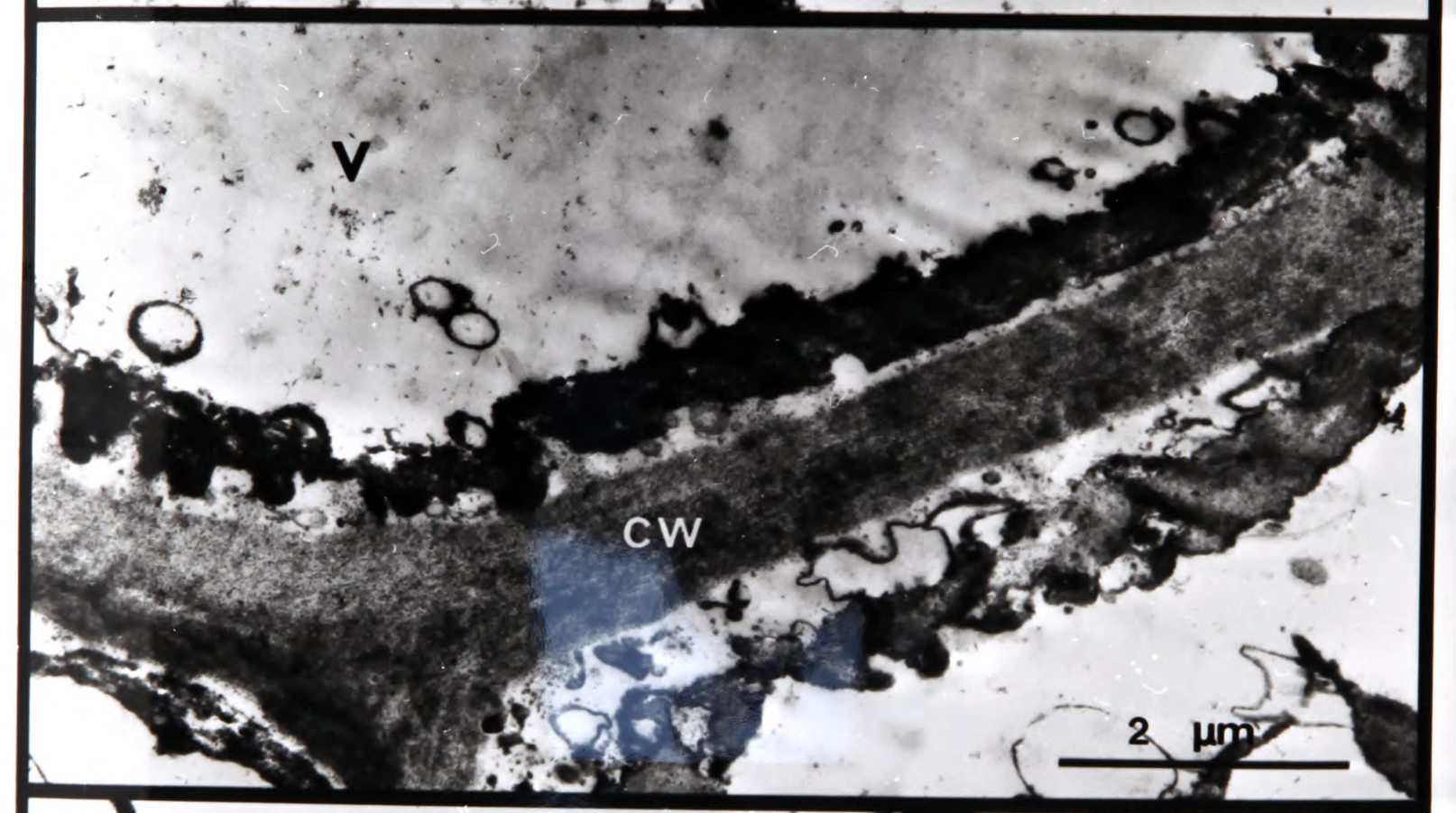
Figure 4.10 Parts of 2 outer gland cells from tissue boiled 15 min before being incubated 90 min for ATPase activity. Note how the membranes have vesiculated and the lack of definition in the cytoplasm.

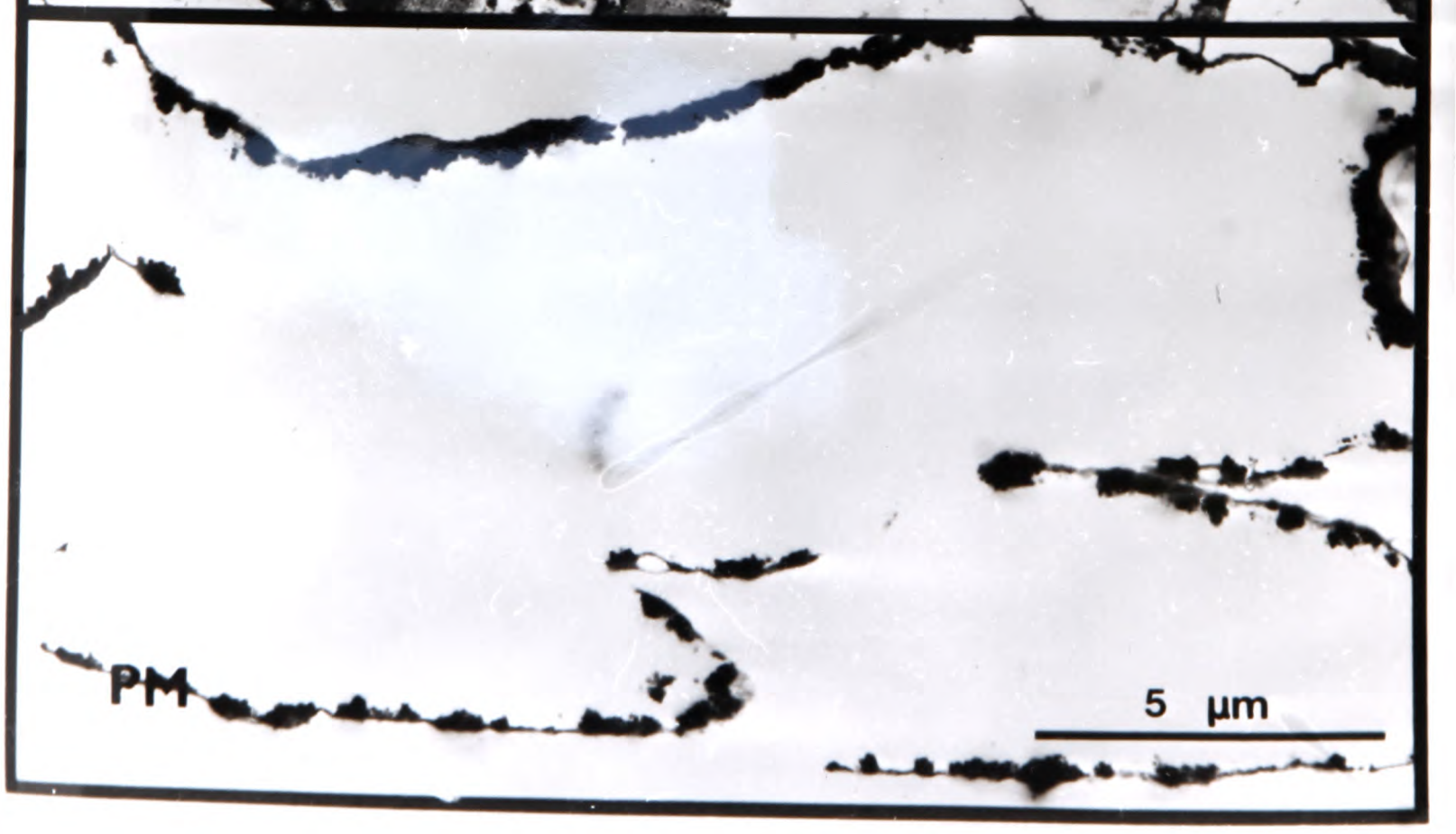
Unstimulated tissue: Spurr's resin: lead citrate poststain. x 15,840.

Figure 4.11 Part of an epidermal cell from tissue incubated 180 min for ATPase activity. The cell has plasmolysed but activity has still been retained in the plasmalemma. Note how the lead II phosphate deposits are to the cytoplasmic side of the membrane.

Unstimulated tissue: Spurr's resin: uranyl acetate - lead citrate poststain. x 7,720.


N
M
V
1 µm


V
CW
2 µm


PM
5 µm

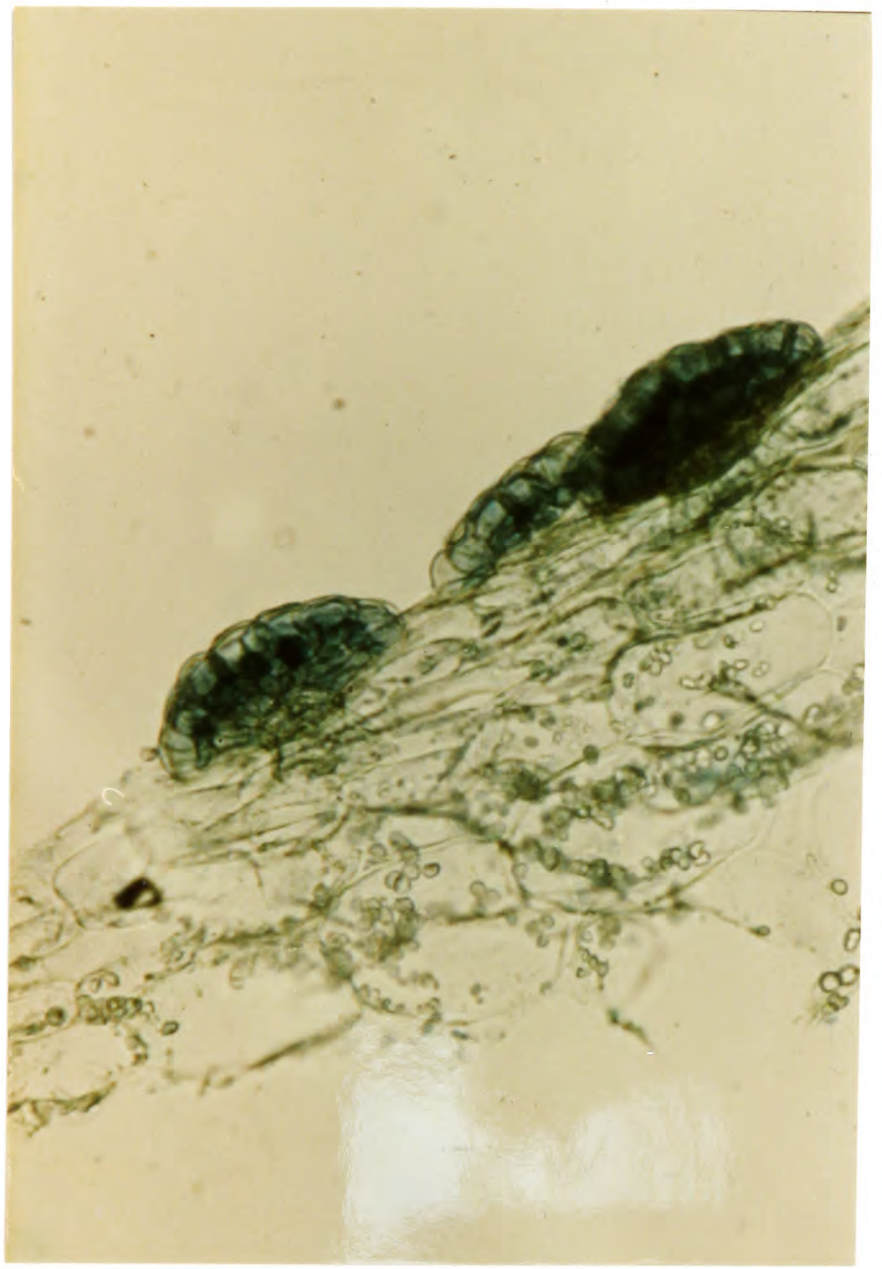


Figure 4.12 Light micrograph showing the staining of leaf material from Dionaea with mercury II - bromophenol blue. Fresh tissue. x 120.

Figure 4.13 Parts of 2 outer secretory cells stained 90 min with mercury II - bromophenol blue. Note particularly the heavy deposits within all the vacuoles and the dark staining of the primary cell wall.

Unstimulated tissue: fixed glutaraldehyde: postfixed mercury II chloride - bromophenol blue only: Spurr's resin: no poststaining. x 16,080.

Figure 4.14 Parts of 2 outer secretory cells stained as Fig. 4.13. Note the weak staining of the endoplasmic reticulum and the strong staining of the mitochondria. Note also the contrast in staining between the primary and labyrinthine cell walls.

Tissue prepared as Fig. 4.14. x 11,250.

Figure 4.15 Parts of 2 outer secretory cells stained as Fig. 4.13 showing the staining of the 2 cell wall layers. Note the strong staining of the plasmodesmata.

Tissue prepared as Fig. 4.14. x 16,220.

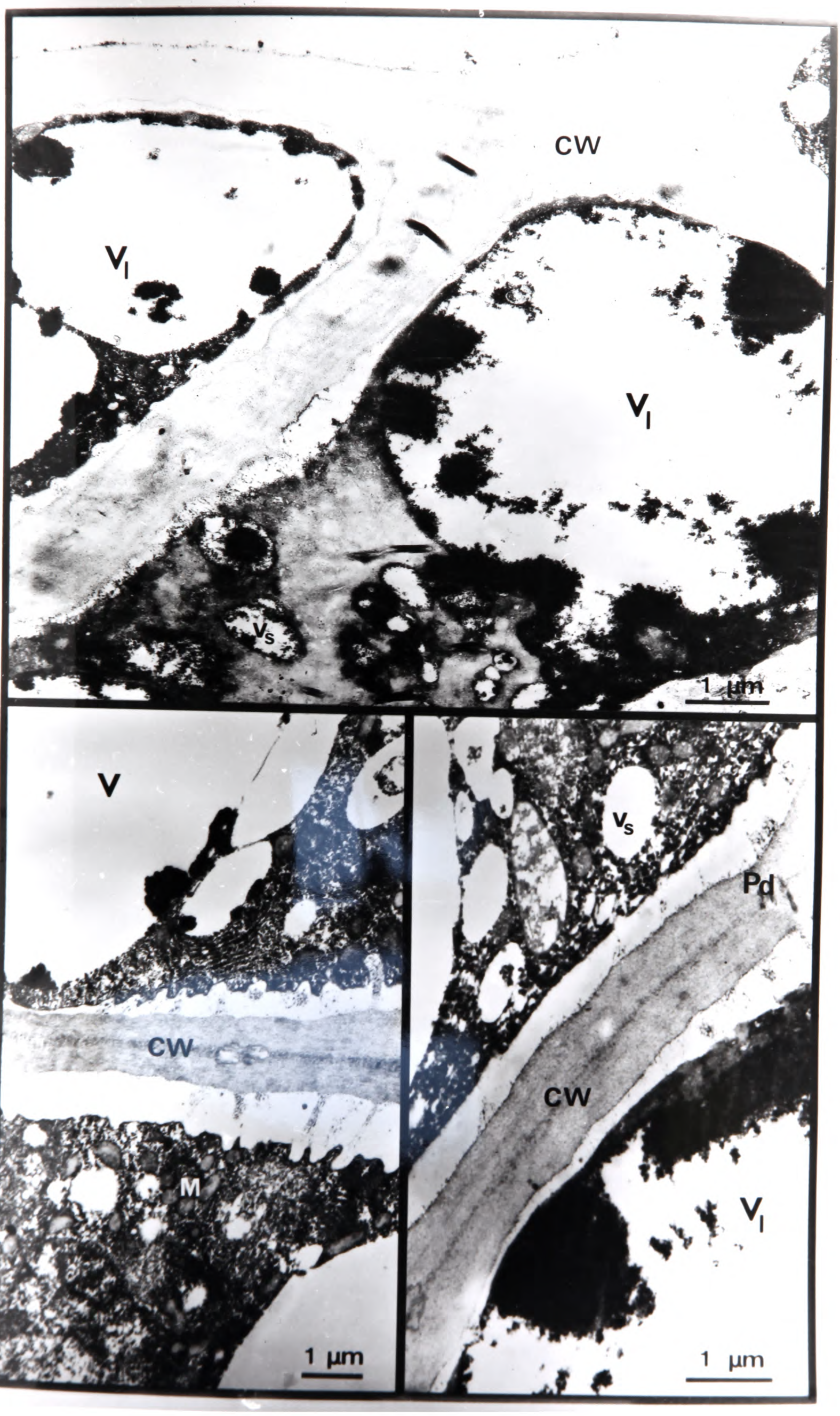

CW
V_l
V_l
V_s
1 µm
V
CW
M
1 µm
V_s
Pd
CW
V_l
1 µm

Figure 4.16 Detail from the cytoplasm of an outer secretory cell showing that comparatively little damage has been done to the organelles by digestion of the thin-section for 90 min with pronase solution.

Unstimulated tissue: Epikote-812: lead citrate post-stain. x 64,410.

Figure 4.17 Detail from the cytoplasm of a stalk cell showing the lipid droplets after 90 min digestion of the thin-section by pronase. Note how little effect this has had compared to digestion with lipase (Fig. 3.12) but that the droplets have lost their sharp, single outline.

Unstimulated tissue: Epikote-812: lead citrate post-stain. x 27,650.

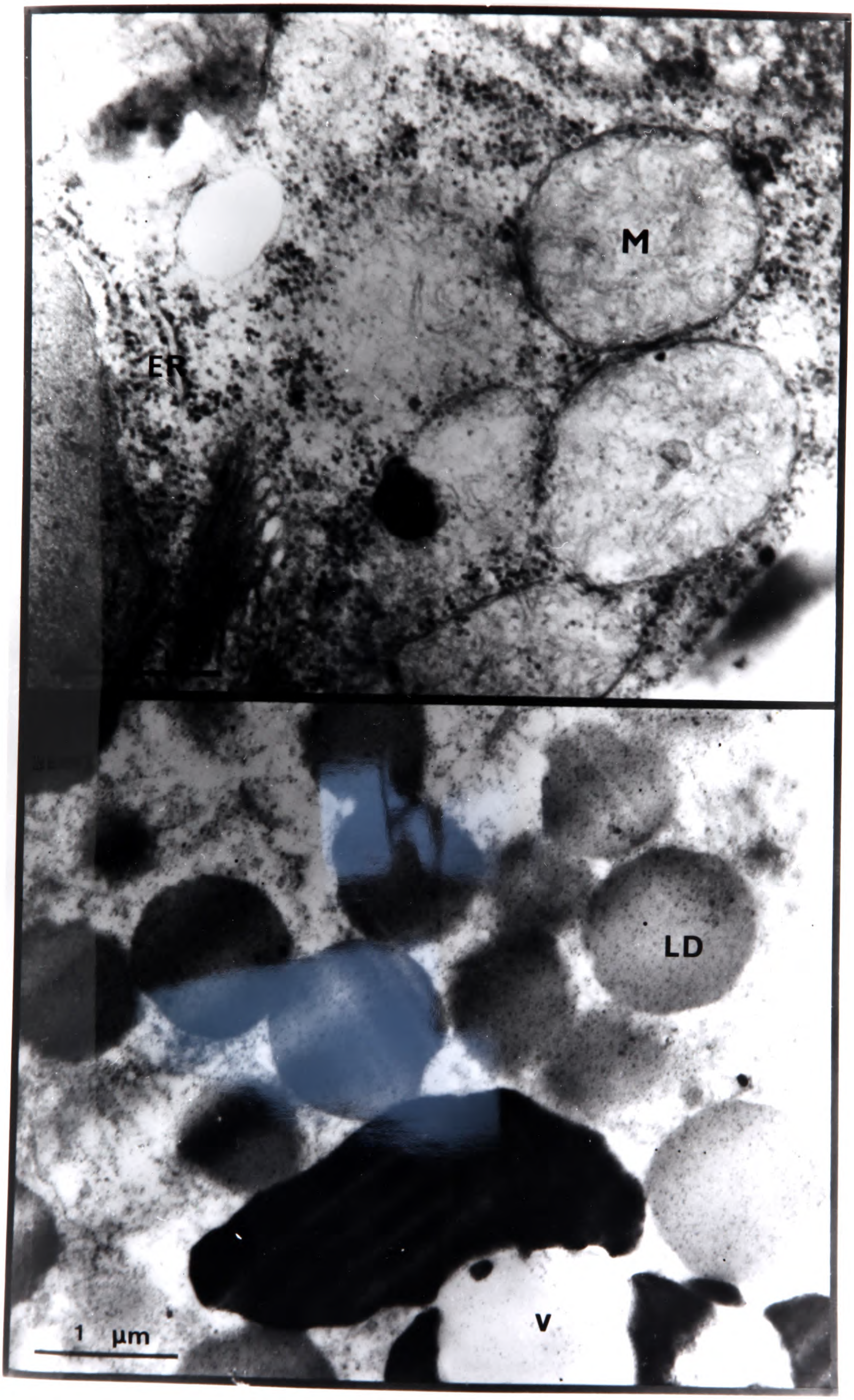

M
ER
LD
V
1 µm

Figure 4.18 Section of parts of outer and inner secretory cells which have been treated 90 min with pronase solution in the thin section. There is an overall loss of contrast throughout the cell but particularly within the vacuoles. It is not, however, complete.

Unstimulated tissue: Epikote-812 resin: no poststaining: exposed 4 s: printed 5 s/f 11 on grade 2 paper. x 6,340.

Figure 4.19 Section showing parts of inner secretory cells and a stalk cell from a control section for Figs. 4.18 : 4.19 : 4.20. Identical material treated without any enzymes present.

Unstimulated tissue: Epikote-812 resin: no poststaining: exposed 4 s: printed 5 s/f 11 on grade 2 paper. x 5,960.

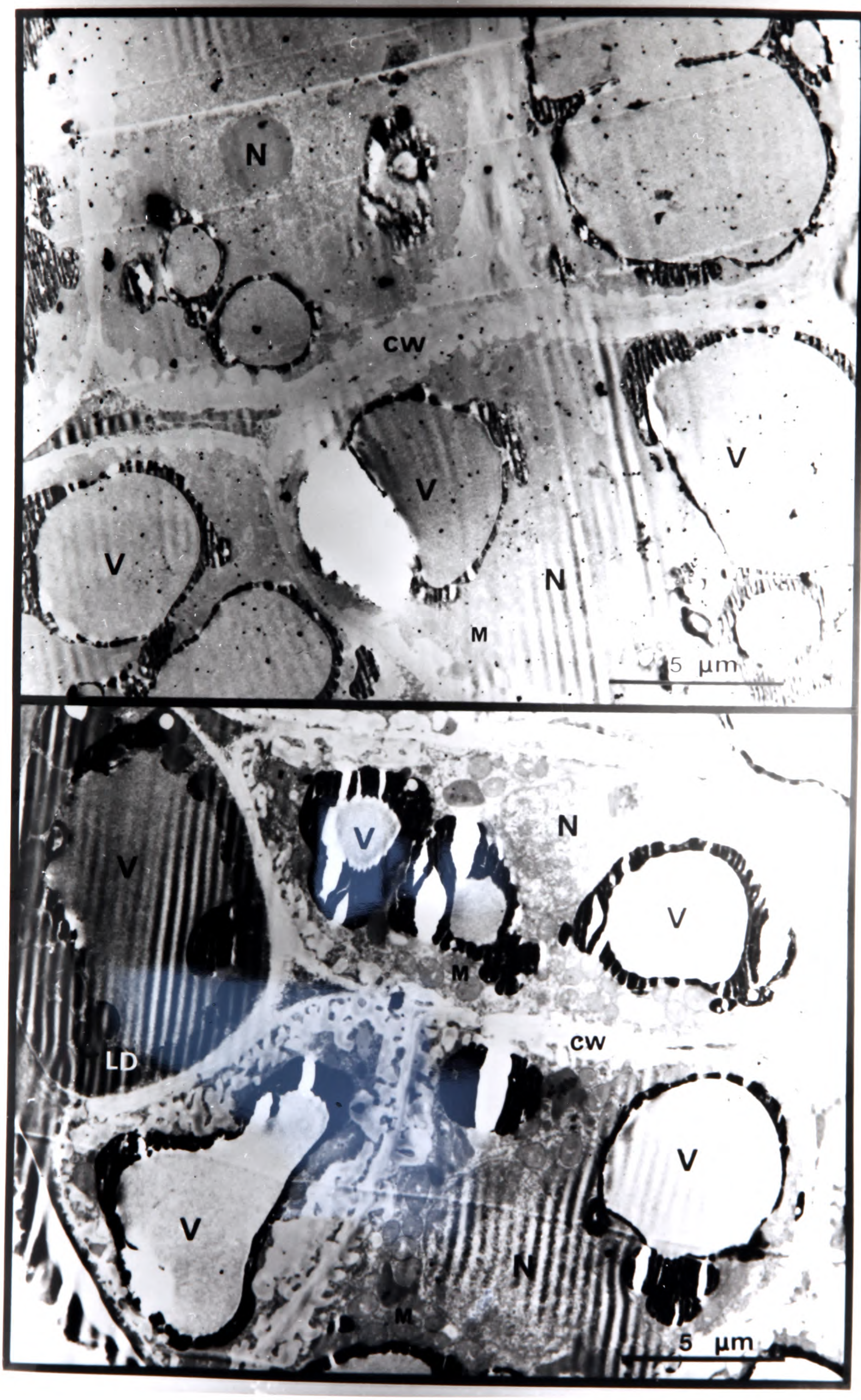

N
CW
V
V
V
N
M
5 µm
V
V
N
V
M
LD
CW
V
V
N
M
5 µm

Figure 4.20 Section showing 2 outer secretory cells treated 90 min with lipase solution. Note how little effect this has had on the intensity of the stain in the vacuoles.

Unstimulated tissue: Epikote-812 resin: no poststain: exposed 4 s: printed 5 s/f 11 on grade 2 paper. x 5,800.

Figure 4.21 Section of part of an outer secretory cell treated 90 min with a solution containing both pronase and lipase (both at half the concentrations given in 2:II:8). Note how the contents of the cytoplasm have been largely destroyed while the staining of the vacuoles is no less than with pronase alone.

Unstimulated tissue: Epikote-812 resin: no poststain: exposed 4 s: printed 5 s/f 11 on grade 2 paper. x 10,200.

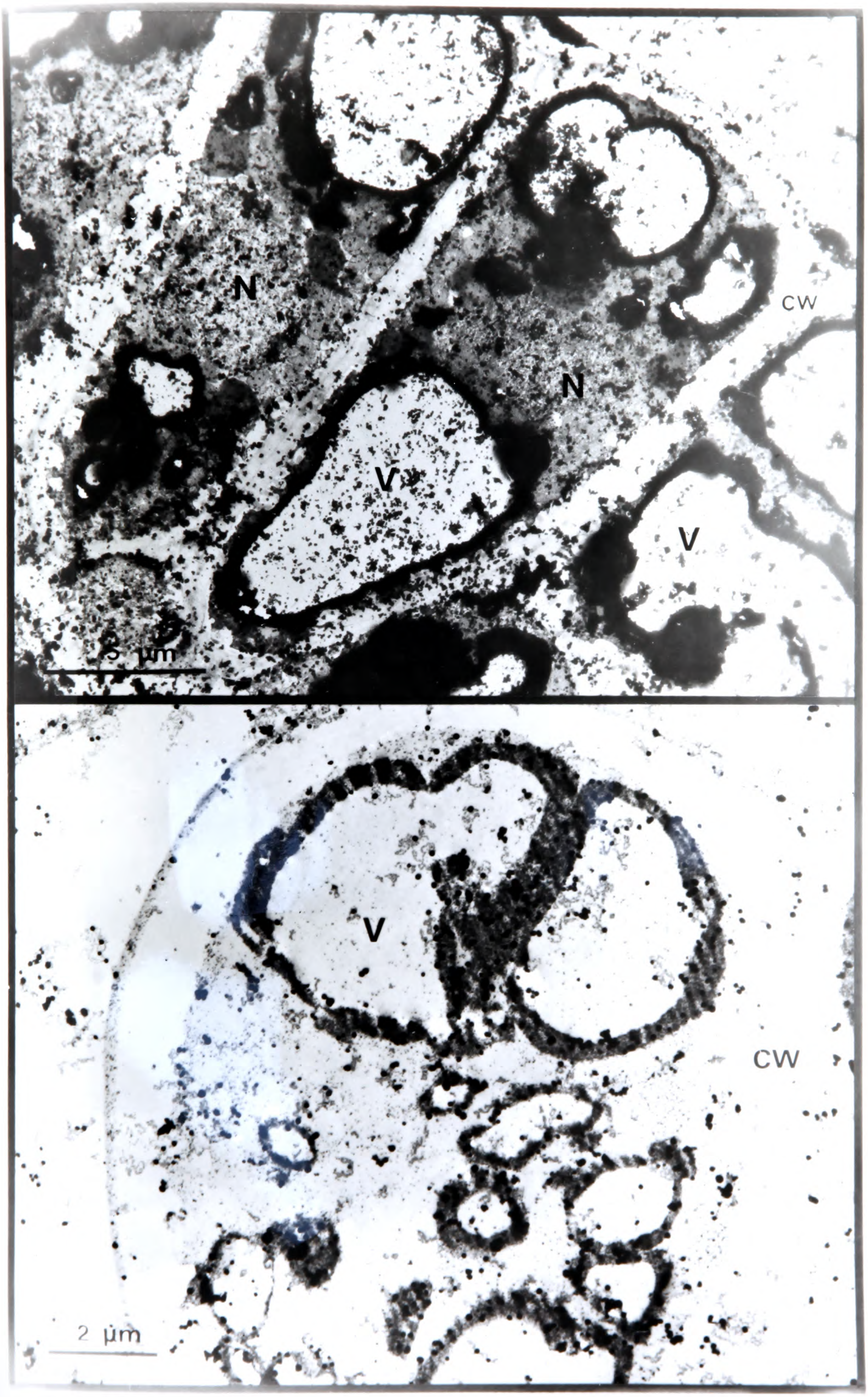

N
N
V
V
CW
3 µm
V
CW
2 µm



Figure 4.22 Light micrograph showing the staining of a digestive gland with iron III chloride.

Fresh tissue. x 300.

Figure 4.23 Longitudinal section through a secretory gland stained with iron II sulphate, but without osmium. Note the dense deposit within the vacuoles.

Unstimulated tissue: fixed glutaraldehyde - iron II sulphate: Epikote-812: lead citrate poststain. x 3,700.

Figure 4.24 Transverse section through a secretory gland without poststaining. Control section for Figs. 4.13 - 4.15 and Fig. 4.23.

Unstimulated tissue: fixed glutaraldehyde only: Epikote-812: lead citrate poststain. x 5,370.

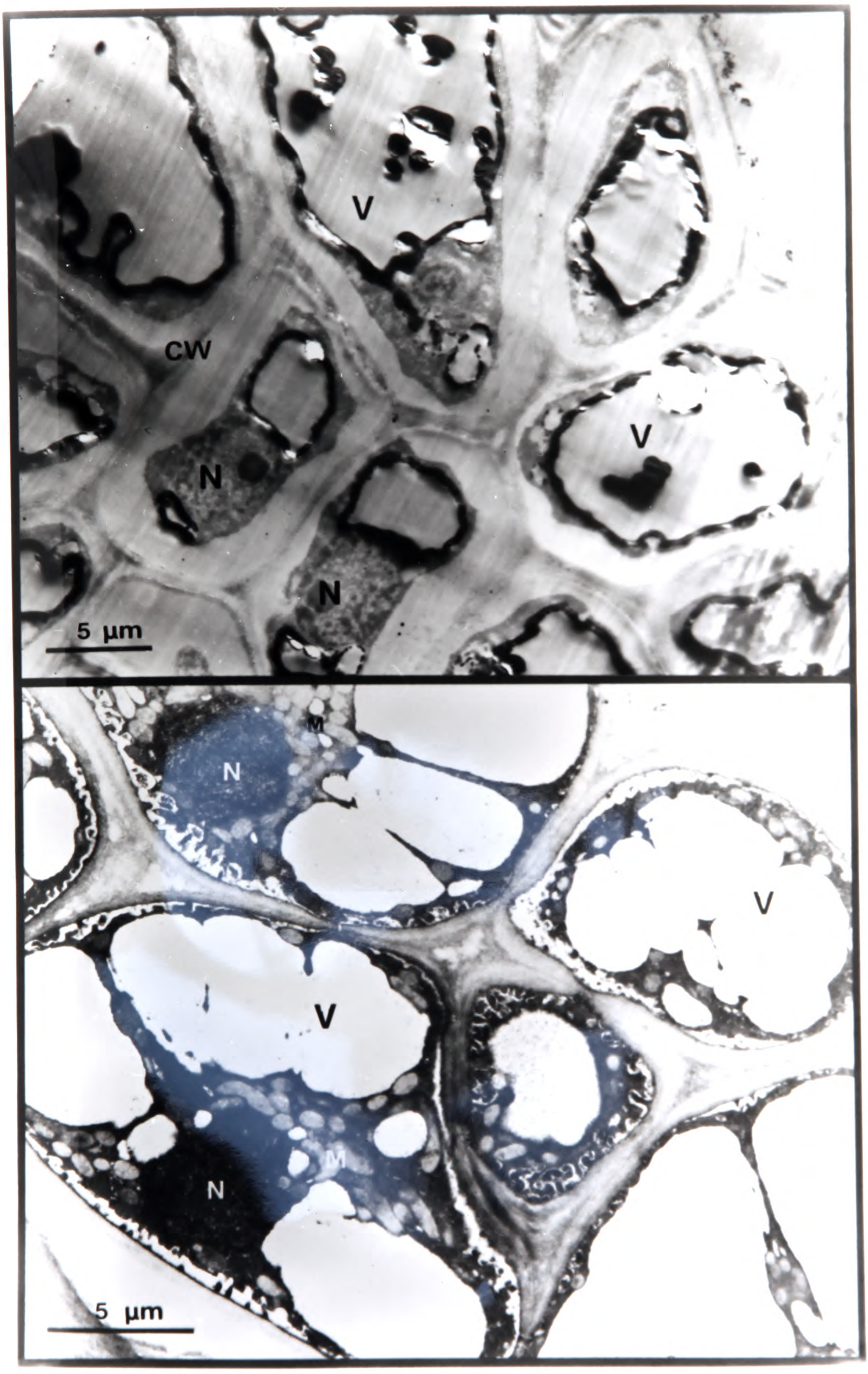

V
CW
N
V
N
5 µm
N
M
V
V
M
N
5 µm

Figure 4.25 Montage autoradiograph showing a cross-section of a gland from tissue fixed a few days before maturation, which has been prepared from plants fed L-[4,5-^{3}H]-leucine for 14 days via other, mature traps. It shows the low silver grain distribution in these autoradiographs, the results of which are given in Tables 4.1 and 5.3. Note how the stalk and basal cells have not yet acquired their mature appearance and how none of the vacuoles in the secretory cells are strongly osmiophilic. Leaves at this stage of development show very little bright red coloration.

Immature, unstimulated tissue: Spurr's resin: uranyl acetate - lead citrate poststained. x 8,980.

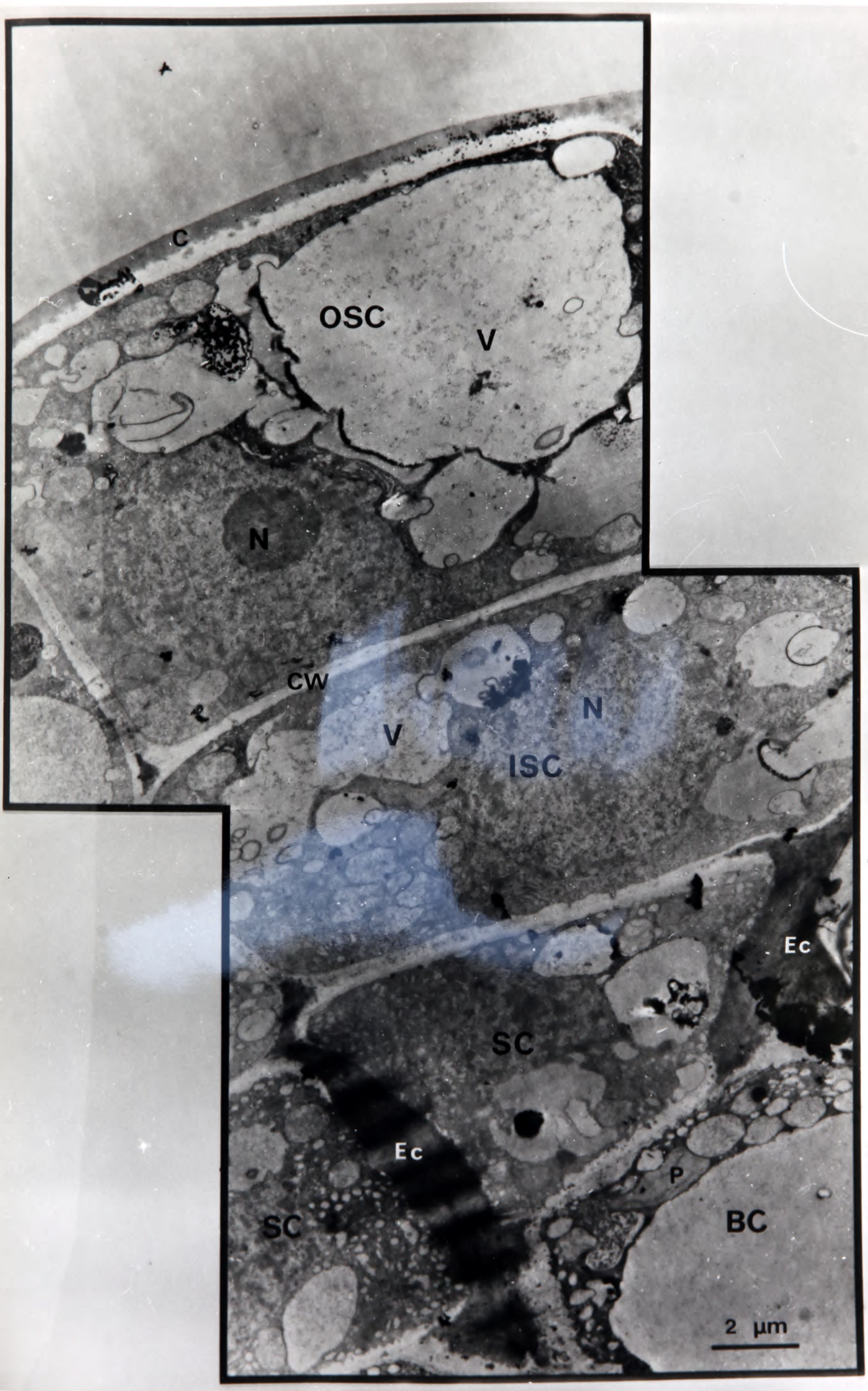

C
OSC
V
N
CW
V
N
ISC
Ec
SC
Ec
P
SC
BC
2 µm

Figure 4.26 Detail from an autoradiograph of a mature outer secretory cell from tissue fed as in Fig. 4.25 showing silver grains located over large and small vacuoles and smooth endoplasmic reticulum.

Unstimulated tissue: Spurr's resin: uranyl acetate - lead citrate poststain. x 45,220.

Figure 4.27 Detail from an autoradiograph of a mature secretory cell from tissue fed as in Fig. 4.25 showing silver grains located over the small vacuoles. Note the numerous profiles of smooth endoplasmic reticulum.

Unstimulated tissue: Spurr's resin: uranyl acetate - lead citrate poststain. x 53,690.

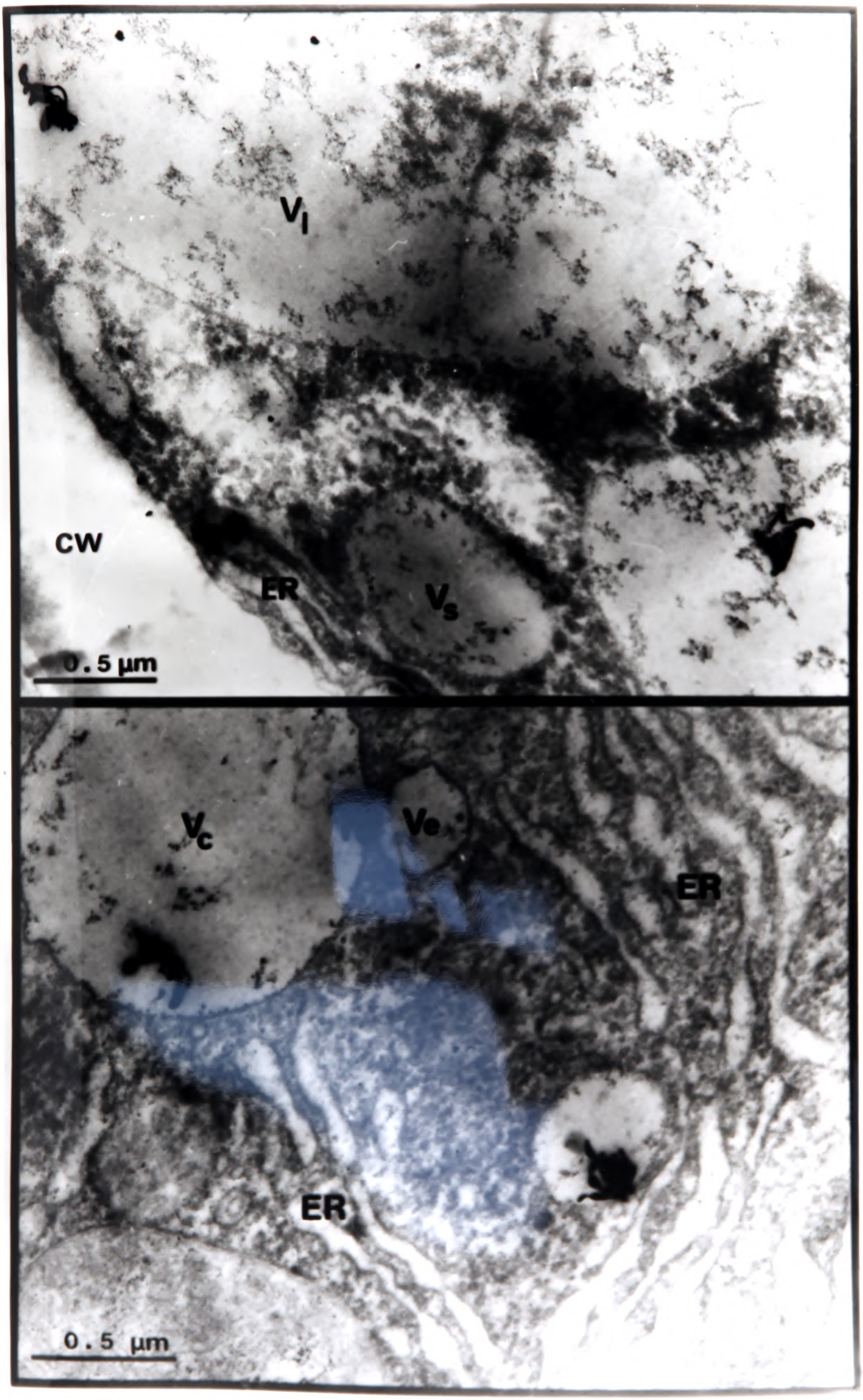

V_l
CW
ER
V_s
0.5 µm
V_c
Ve
ER
ER
0.5 µm

Figure 4.28 Detail from an autoradiograph of immature tissue (as in Fig. 4.25) showing parts of a stalk cell (lower left) and basal cell (top) and of the endocuticle (✪). Note that radiolabel is associated with small vesicles that are fusing with the plasmalemma (arrows).

Immature, unstimulated tissue: Spurr's resin: uranyl acetate - lead citrate poststain. x 24,380.

Figure 4.29 Detail from an autoradiograph of immature tissue (as in Fig. 4.25) showing part of a stalk cell in which a mitochondrion and a dictyosomal vesicle are labelled.

Immature, unstimulated tissue: Spurr's resin: uranyl acetate - lead citrate poststain. x 57,260.

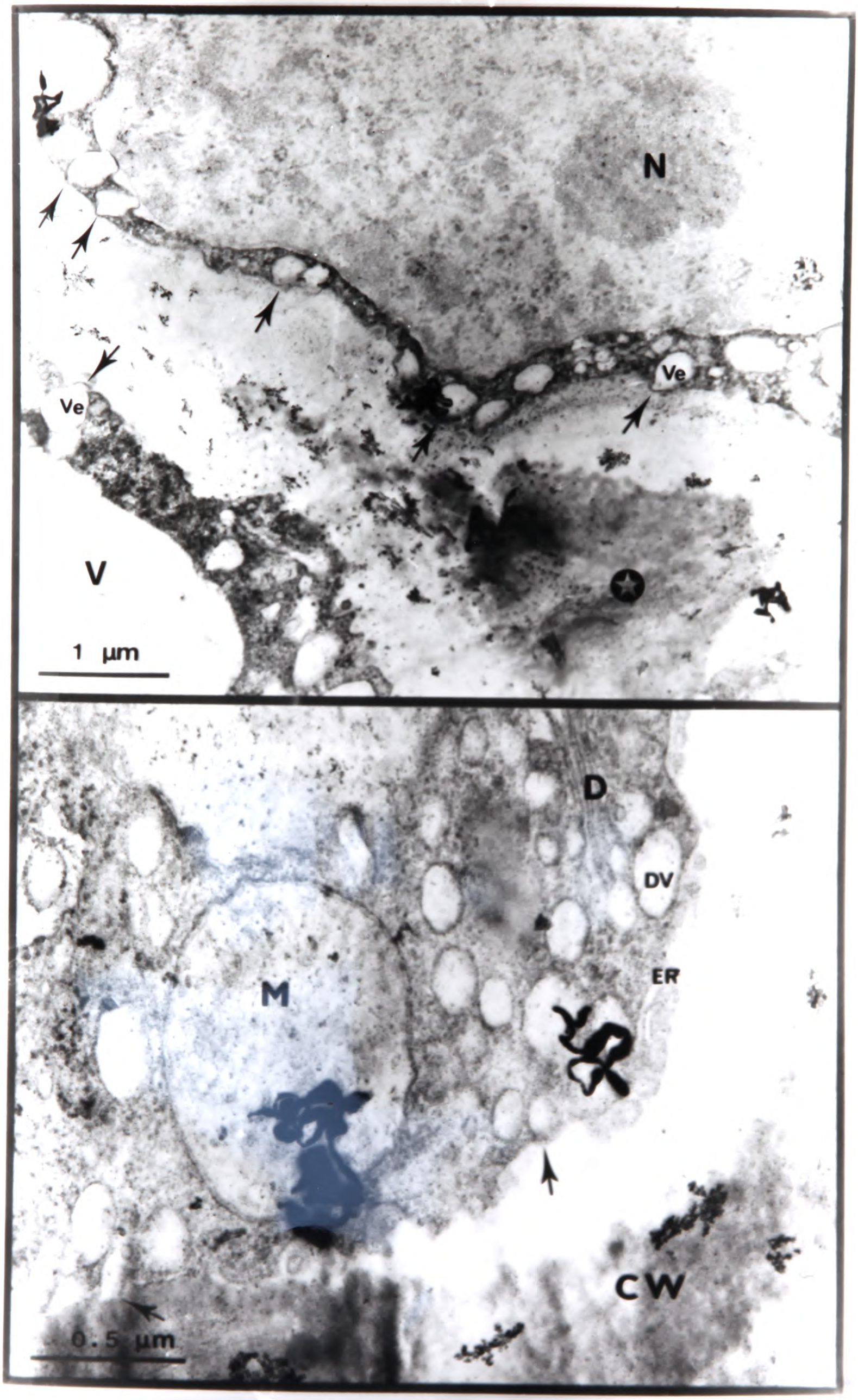

N
Ve
Ve
V
1 µm
D
DV
ER
M
CW
0.5 µm

Figure 4.30 Detail from an autoradiograph of immature tissue (as in Fig. 4.25) showing radiolabel located in the small vacuole and in vesicles associated with smooth endoplasmic reticulum in an outer secretory cell.

Immature, unstimulated tissue: Spurr's resin: uranyl acetate - lead citrate poststain. x 44,900.

Figure 4.31 Detail from an autoradiograph of immature tissue (as in Fig. 4.25) showing radiolabel located in the smooth endoplasmic reticulum of an inner secretory cell.

Immature, unstimulated tissue: Spurr's resin: uranyl acetate - lead citrate poststain. x 105,600.

Figure 4.32 Detail from an autoradiograph of immature tissue (as in Fig. 4.25) showing a silver grain overlying endoplasmic reticulum with both rough and smooth membranes in an outer secretory cell.

Immature, unstimulated tissue: Spurr's resin: uranyl acetate - lead citrate poststain. x 110,400.

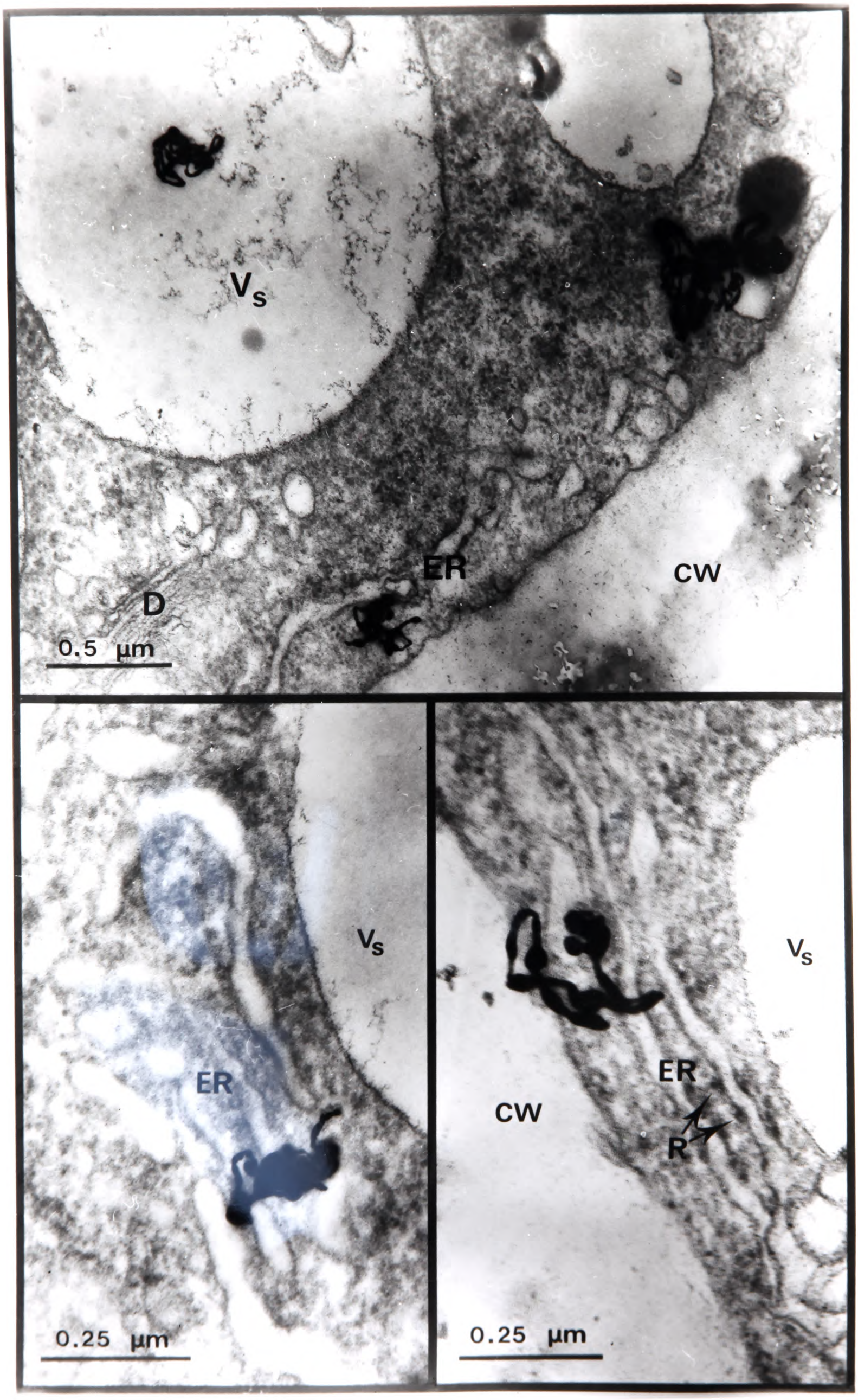

Vs
ER
CW
D
0.5 µm
Vs
ER
0.25 µm
Vs
ER
CW
R
0.25 µm

Figure 4.33 Isopyknic ultracentrifugation gradient showing the relative buoyant densities of acid phosphatase in secretion collected from traps fed with 4%(w/v) bacto-peptone in 50%(v/v) $^{2}H_{2}O$ solution (Δ——Δ) and the control traps fed with 4%(w/v) bactopeptone in $^{1}H_{2}O$ solution (▲——▲). Fractions are plotted as the refractive index shift relative to an internal marker of β-galactosidase (ρ=1.300 kg l^{-1}). Refractive index values of the β-galactosidase peaks are:

50%(v/v) $^{2}H_{2}O$: n^{23} = 1.3624

control : n^{23} = 1.3627

Peak enzyme activity values are:

50%(v/v) $^{2}H_{2}O$ = 47.8 pkat

control = 62.8 pkat

Tris/acetic acid buffer (pH6.0) at 42,000 rev min^{-1} (110,000 g) for 40 h at 5.5°C.

a) Gradient relative to β-galactosidase

b) Normal-probability distribution plot to examine the shape of the curve.

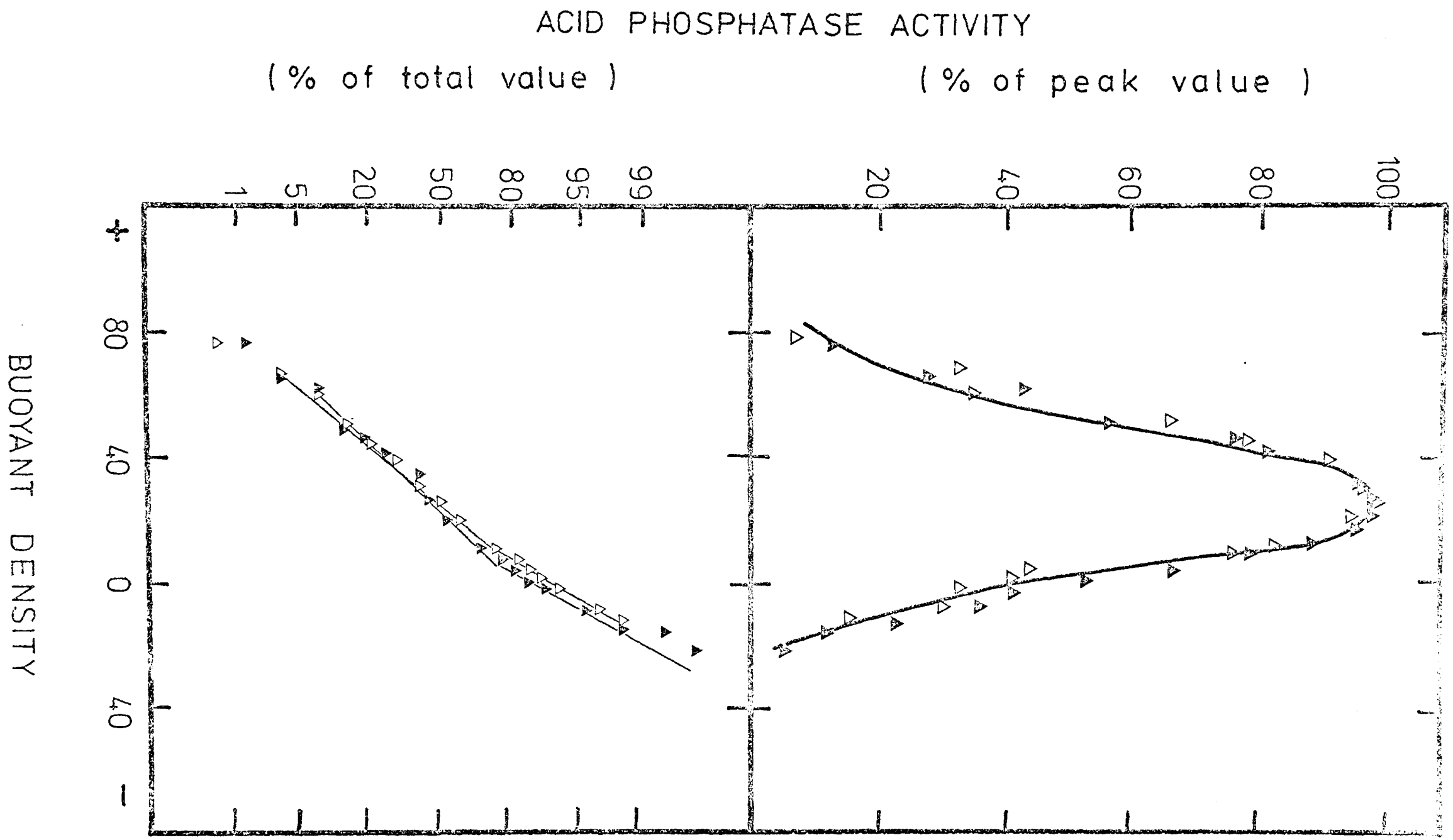

ACID PHOSPHATASE ACTIVITY
(% of total value)
(% of peak value)
1
5
20
50
80
95
99
20
40
60
80
100
+
80
40
0
40
BUOYANT DENSITY

Figure 4.34 Isopyknic ultracentrifugation gradient showing the relative buoyant densities of acid phosphatase in secretion collected from traps fed with 4%(w/v) bacto-peptone in 80%(v/v) $^{2}H_{2}O$ solution (△——△) and the control traps fed with 4%(w/v) bactopeptone in $^{1}H_{2}O$ solution (▲——▲). Fractions are plotted as the refractive index shift relative to an internal marker of β-galactosidase (ρ=1.300 kg l^{-1}). Refractive index values of the β-galactosidase peak are:

80%(v/v) $^{2}H_{2}O$: n^{24} = 1.3622

control : n^{24} = 1.3619

Peak enzyme activity values are:

80%(v/v) $^{2}H_{2}O$ = 61.0 pkat

control = 100.0 pkat

Tris/acetic acid buffer (pH6.0) at 42,000 (110,000 g) rev min^{-1} for 46 h at 5.5°C.

a) Gradient relative to β-galactosidase.

b) Normal-probability distribution plot to examine the shape of the curve.

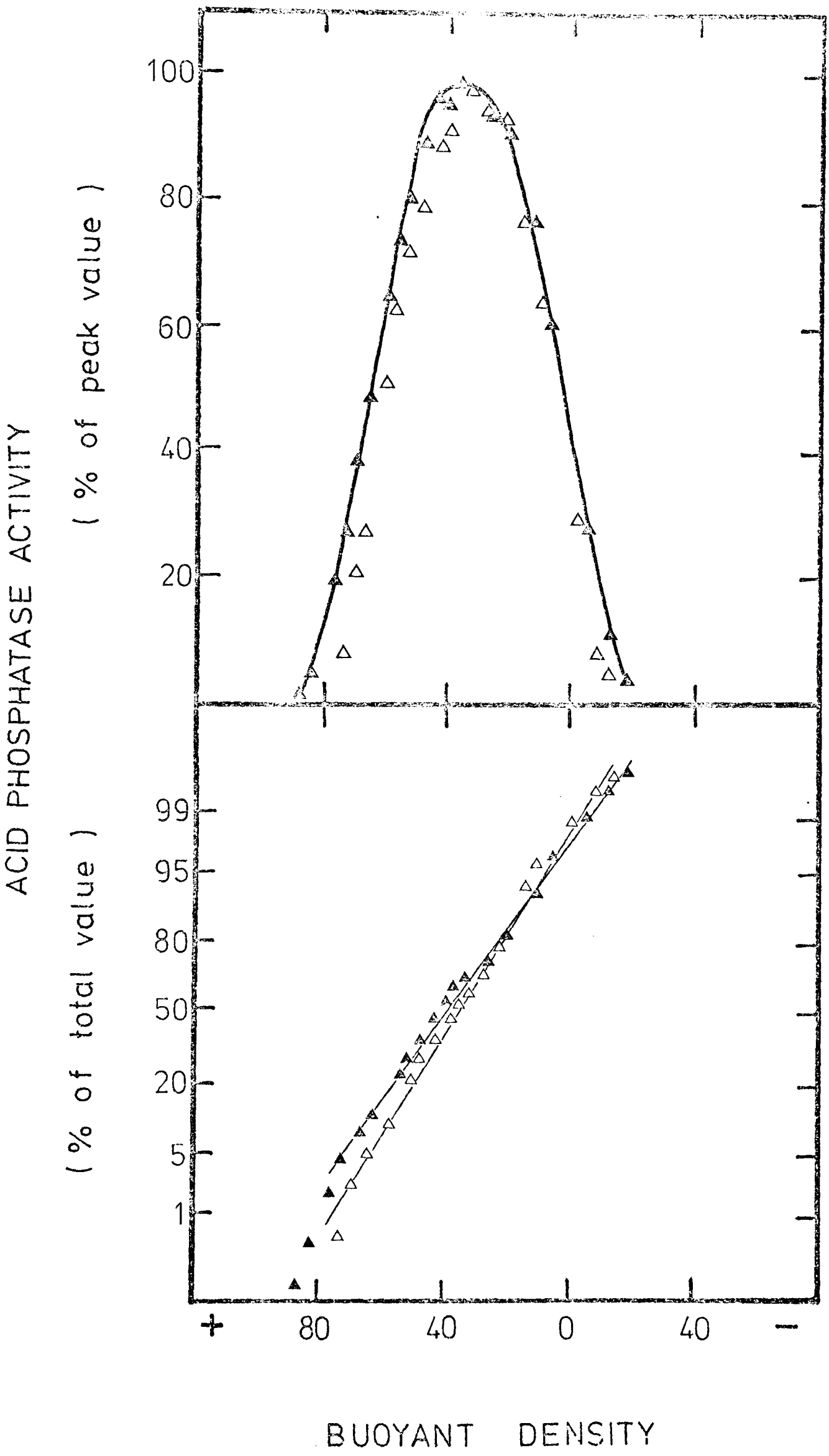

ACID PHOSPHATASE ACTIVITY
(% of peak value)
100
80
60
40
20
(% of total value)
99
95
80
50
20
5
1
+
80
40
0
40
−
BUOYANT DENSITY

Figure 4.35 Elution profiles from Sephadex G-25 chromatography of secretion collected from 15 traps stimulated for 4 days with 4%(w/v) bactopeptone solution containing 1.125 μCi ml^{-1} L-[U-^{14}C]-leucine showing the effect of precipitation with 80%(v/v) ethanol on the distribution of:

a) Absorption at 280 nm;

b) Acid phosphatase activity;

c) Radioactivity.

▲ = control (0.5 ml secretion + 2.0 ml running buffer)

Δ = treatment (0.5 ml secretion + 2.0 ml ethanol left for 20 min at room temperature and then centrifuged for 5 min)

Running buffer was 50 mM-sodium acetate (pH5.1), and 28-drip (approx. 1.0 ml) fractions were collected. 0.5 ml of secretion from day 4 after stimulation was used.

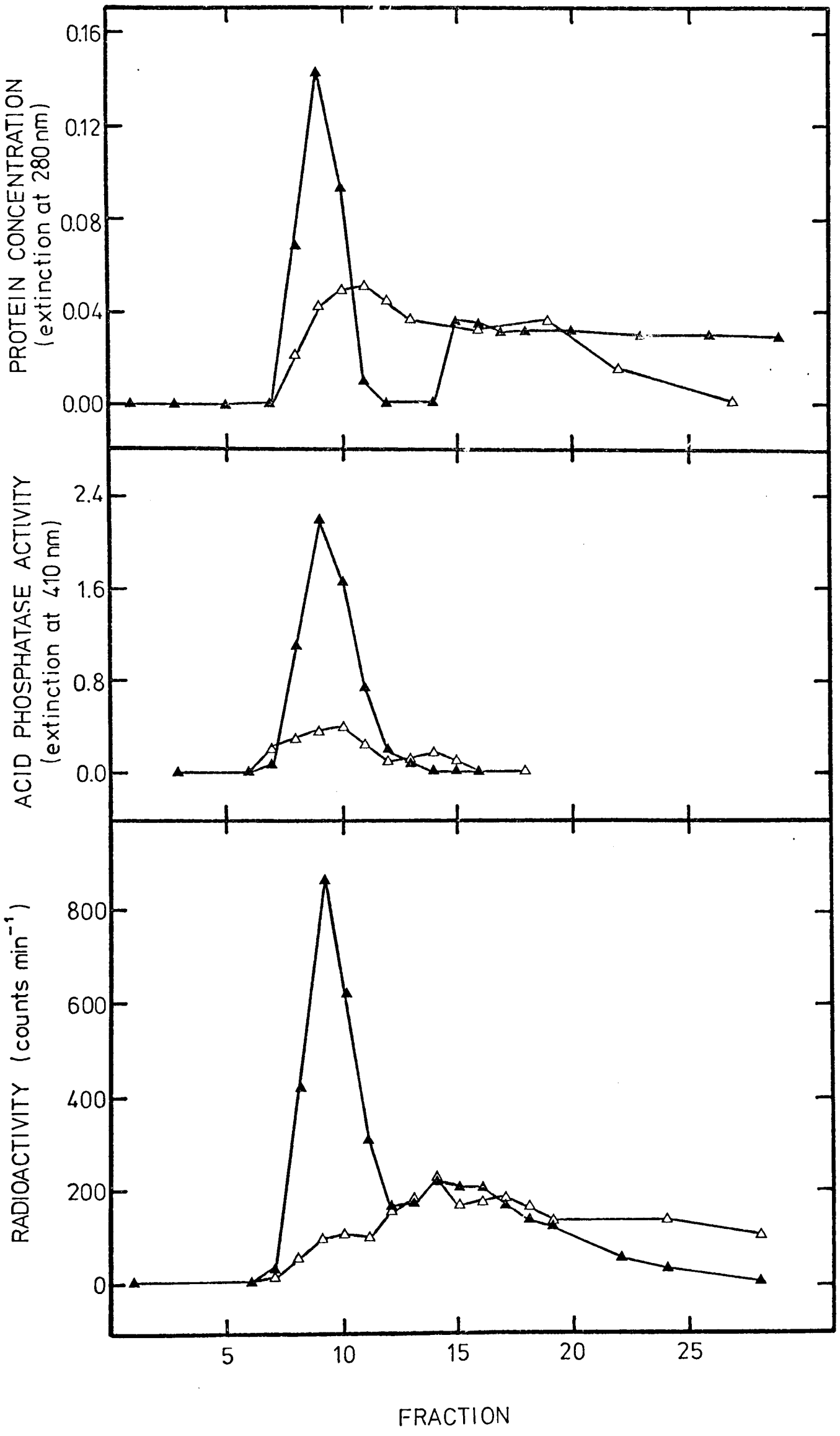

PROTEIN CONCENTRATION
(extinction at 280 nm)
0.16
0.12
0.08
0.04
0.00
ACID PHOSPHATASE ACTIVITY
(extinction at 410 nm)
2.4
1.6
0.8
0.0
RADIOACTIVITY (counts min^{-1})
800
600
400
200
0
5
10
15
20
25
FRACTION

Figure 4.36 Elution profiles from Sephadex G-25 chromatography of secretion collected from traps stimulated over 7 days with 4%(w/v) bactopeptone solution containing:

- ● 5 μCi ml^{-1} L-[4,5-^{3}H]-leucine
- ○ 5 μCi ml^{-1} L-[4,5-^{3}H]-leucine + 0.05%(w/v) benlate + 1000 I.U. ml^{-1} benzyl penicillin

a) Absorption at 280 nm;

b) Acid protease activity;

c) Acid phosphatase activity;

d) Radioactivity.

Running buffer was 50 mM-sodium acetate (pH5.0) and 28-drip (approx. 1.0 ml) fractions were collected. 1.0 ml of secretion mixed from all of the 7 days collected was applied to the column. There were 8 traps used for each treatment.

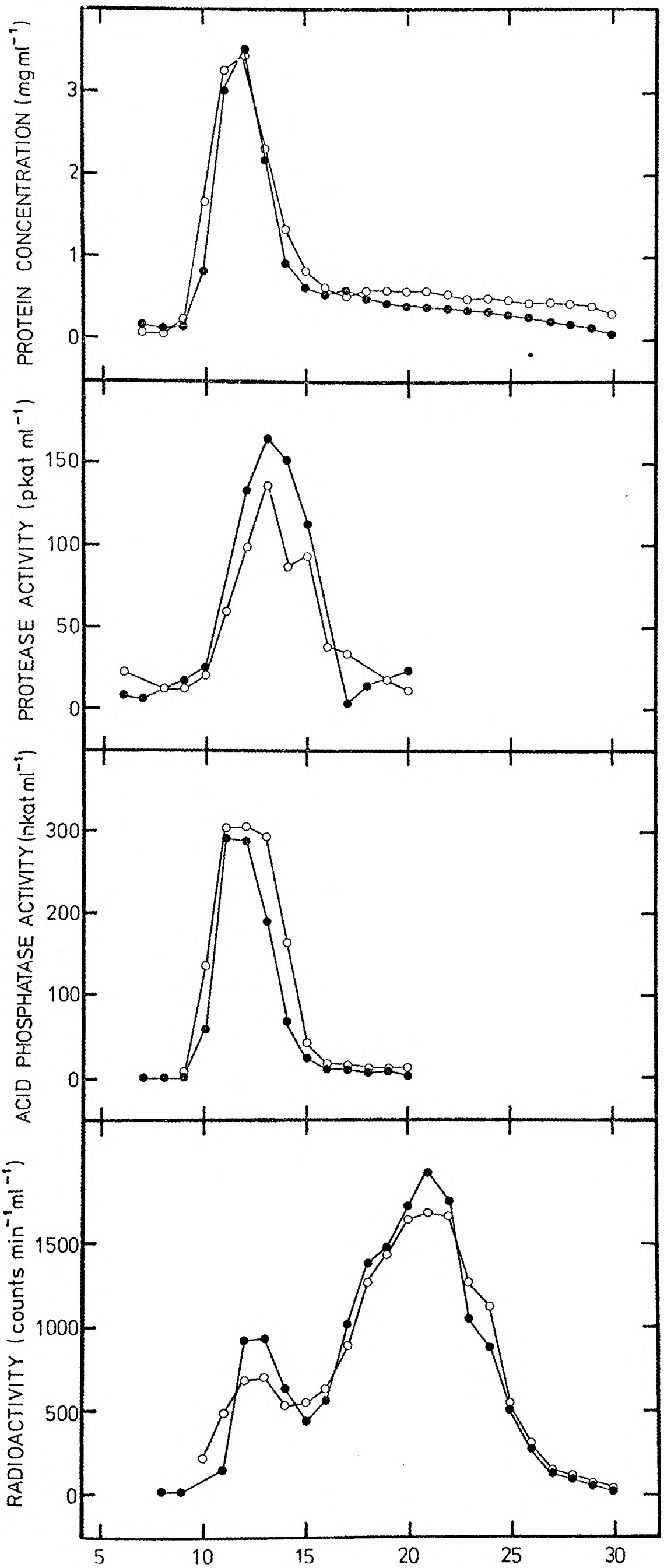

PROTEIN CONCENTRATION (mg ml⁻¹)
0
1
2
3
PROTEASE ACTIVITY (pkat ml⁻¹)
0
50
100
150
ACID PHOSPHATASE ACTIVITY (nkat ml⁻¹)
0
100
200
300
RADIOACTIVITY (counts min⁻¹ ml⁻¹)
0
500
1000
1500
5
10
15
20
25
30

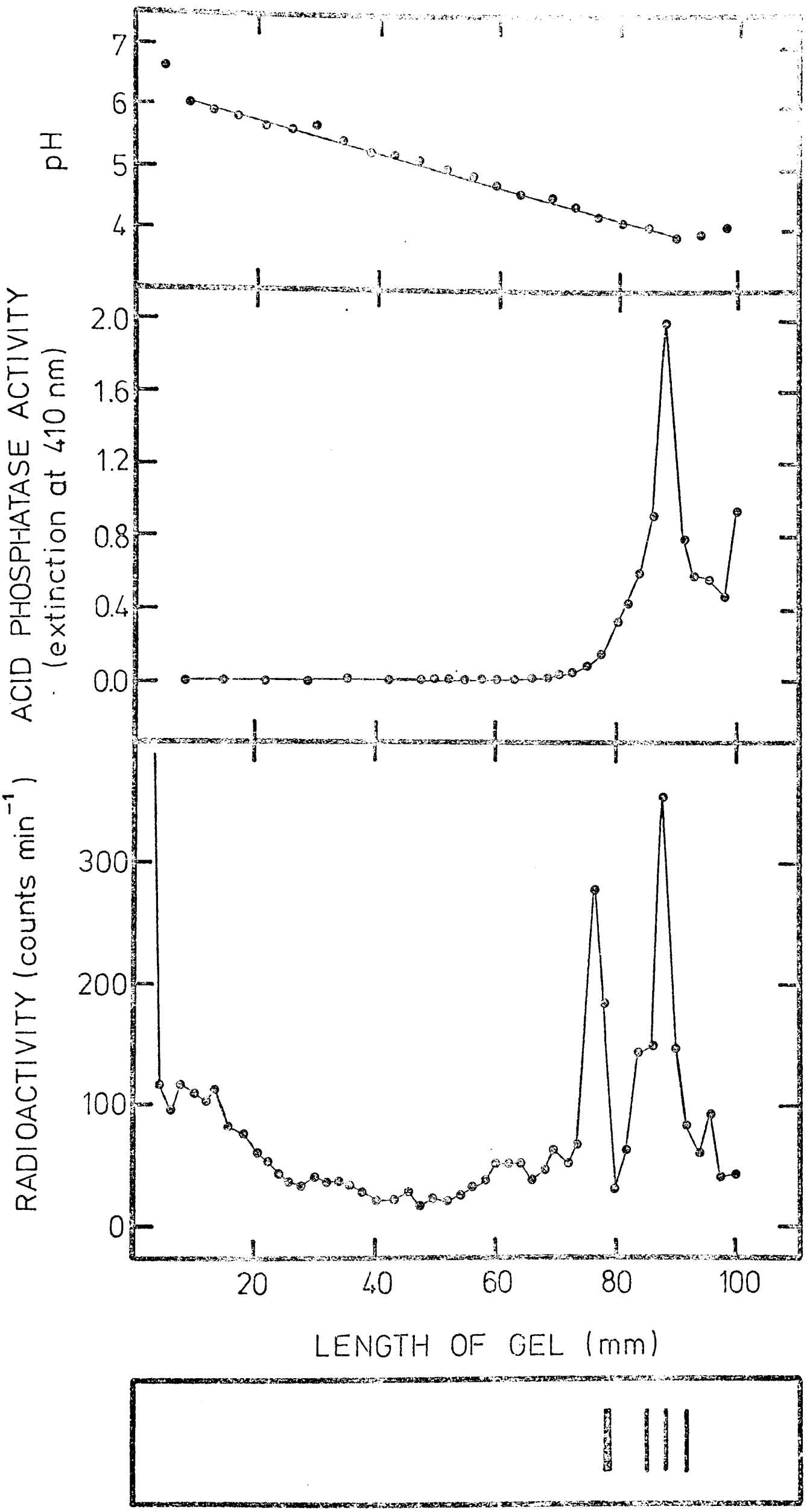
Figure 4.37 Isoelectric focussing profile of secretion collected from 13 traps stimulated with 4%(w/v) bactopeptone solution containing 1.125 μCi ml^{-1} L-[U-^{14}C]-leucine. 1.2 ml crude secretion was mixed with 120 mg sucrose to give a 10% (w/v) sucrose solution. 400 μl of this was placed onto each of 3 gel tubes, and 400 μl 10%(w/v) sucrose in water placed onto a fourth gel tube. Isoelectric focussing was conducted at 350 V for 160 min followed by 700 V for 40 min. Gels were then extracted and treated as described in the Methods (2:IV:3).

a) pH gradient;

b) Acid phosphatase activity;

c) Radioactivity;

d) Total protein.


pH
7
6
5
4
ACID PHOSPHATASE ACTIVITY
(extinction at 410 nm)
2.0
1.6
1.2
0.8
0.4
0.0
RADIOACTIVITY (counts min^{-1})
300
200
100
0
20
40
60
80
100
LENGTH OF GEL (mm)

CHAPTER 5

DISCHARGE OF THE SECRETION

I INTRODUCTION

II RESULTS

1) Stimulant Feeding Experiments

2) Autoradiography

III DISCUSSION

1) General Aspects of the Discharge of Secretion

2) Ultrastructural Features of the Discharge of Secretion

3) The Effects of Colchicine, Cytochalasin B and Cycloheximide

I INTRODUCTION

The classic works on a stimulus-induced discharge of a secretion are those of Caro and Palade (1964), Jamison and Palade (1966) and Siekevitz and Palade (1960) which established the mechanism by which hydrolytic enzymes are released in the exocrine pancreas of the dog. They showed that these are released from the zymogen granules by the direct fusion of these granules with the plasma membrane.

In plants, many secretory systems do not function in response to an externally-applied stimulus but respond to the physiological or morphological state of the tissue. Nectaries, salt glands and root-caps, which display several different mechanisms, are not dependent on an external stimulus to induce secretion. Drosophyllum, however, has a comparable secretory mechanism to the root-cap - polysaccharides are synthesized at the dictyosomes and discharged by direct fusion of dictyosomal vesicles with the plasmalemma - but shows greatly increased activity following stimulation (Schnepf, 1961b; 1963a). The stimulus in Drosophyllum is apparently acting to increase the synthetic activity of the secretory cells whereas in Dionaea the situation is more comparable to the exocrine pancreas, with a store of pre-formed secretory material to be released. Again as in the pancreas, this store is shown to be re-charged with new protein as secretion proceeds. From what little has been done, it appears that the protein-secreting glands of other carnivorous plants may have similar response properties to Dionaea although there is no evidence to suggest that the secretory pathways are the same.

Previously, it has been shown that in Dionaea the discharge of secretion occurs only in response to nitrogenous matter within the trap. Darwin (1875) demonstrated that secretion was not induced by a number of inorganic or dried organic materials but that "an extremely small amount of nitrogenous matter is sufficient to excite the glands" (Darwin, 1875). He also showed that continuous stimulation was required to maintain secretion since when the stimulating material (usually albumen, gelatine or roast meat) was removed from the trap, secretion ceased and the trap re-opened within 48 h. Over 100 years later, the subject was further investigated and it was shown that a natural secretory response could be induced by solutions of certain small nitrogenous molecules (Robins, 1975; 1976). More recently, Lichtner and Williams (1977) have claimed that continuous agitation of the trigger hairs is sufficient to induce secretion. They do not, however, demonstrate that the fluid they obtain contains hydrolases and its measured pH of 1.5 - 3.5 is too low for this to be digestive fluid (see Chapter 6 and Scala et al, 1969). Other than these low pH readings, they fail to give any information concerning the fluid they observed. As Robins (1976) has suggested that fluid and protein may be under separate release mechanisms it is possible that Lichtner and Williams (1977) are inducing the release of fluid without the release of hydrolases. This needs further investigation.

The drugs colchicine and cytochalasin B are well documented as having considerable activity against secretory systems in animals (Allison and Davies, 1974). The effect

of either of these drugs cannot be extrapolated from one system to another although in animals colchicine consistently inhibits the release of lysosomal hydrolases while cytochalasin B consistently has the opposite effect (Allison and Davies, 1974 : Tables 2 and 3). The release of α-amylase by rat salivary gland is, however, inhibited by cytochalasin B whereas colchicine has been shown, in separate investigations, to both inhibit and have no effect on this release. Chrispeels (1972) has found that neither drug affects the release of α-amylase by isolated aleurone cells of barley or the incorporation of hydroxy-proline-rich cell wall glycoproteins into the wall of phloem-parenchyma cells in carrot root disc culture. The release of α-amylase in barley aleurone tissue apparently involves the fusion of endoplasmic reticulum-derived vesicles with the plasmalemma and is inhibited by inhibitors of protein synthesis (Vigil and Ruddat, 1973).

II RESULTS

1) Stimulant Feeding Experiments

The rate of release of fluid per unit area of lobe adaxial surface is approximately linear over the first six days when traps are repeatedly stimulated to secrete with 4%(w/v) bactopeptone solution (Figs. 5.1 : 5.3). If, however, the stimulation is with a natural prey, such as a Blue-bottle fly (Calliphora sp.), then the rate of release of fluid increases daily, reaching a peak after six to seven days and then rapidly declining (Fig. 5.2a). Under the same conditions of stimulation the release of protein follows very similar patterns (Figs. 5.2b : 5.4) except that when stimulated with Calliphora the peak of protein concentration is reached after only four days (Fig. 5.2b).

It has previously been shown that the nature of the stimulant administered will profoundly affect the quantity and protein content of the ensuing secretion (Robins, 1975; 1976). In Fig. 5.1, the results from Robins (1976) have been re-plotted as per day following the start of secretion, not, as previously, per calendar day after the first day on which stimulant was administered (see Robins, 1976 : Fig. 1). When displayed in this way, the rates of secretion from traps stimulated with pure small nitrogenous molecules are also shown to be approximately linear and not sigmoidal as previously thought.

When Dionaea lobes are treated separately with either colchicine or cytochalasin B, the linearity of the secretion

of fluid and protein is not affected (Figs. 5.3 : 5.4). Neither is the concentration of protein altered by either drug (Table 5.1). Cytochalasin B, however, causes a 20% elevation in the amount of fluid secreted and hence in the total amount of protein, but colchicine shows no such effect (Figs. 5.3 : 5.4a). DMSO alone has no effect.

Both drugs, however, have a considerable effect on the amount of enzymatic activity in the secretion (Table 5.1). Figs. 5.5 and 5.6 show the secretion profiles for (a) the acid protease and (b) the acid phosphatase content of the secretion. By day 6 the amounts of protease activity released per unit area are depressed to 43.0% and 62.3% of the control when, respectively, colchicine and cytochalasin B are present in the stimulating solution. The amount of acid phosphatase present is slightly greater, there being 76.1% and 69.0% of the control values when, respectively, colchicine and cytochalasin B are exhibited. These observed depressions of enzymatic activity are not due to any direct effect of the drugs on the enzymes, as shown in Table 5.2. Indeed a stimulation of protease activity occurs with both these drugs which is not dose-dependent whereas the acid phosphatase is not affected by either colchicine or cytochalasin B over the range exhibited.

2) Autoradiography

Table 5.3 shows the results of the autoradiographic experiment in which lobes on plants previously loaded with L-[4,5-^{3}H]-leucine were stimulated to secrete (see 2:II:6).

As in Table 4.1 (from which the day 0 data is extracted) the observed distribution of silver grains is given together with the calculated distribution if that same number were randomly spread across the gland.

For each set of results, the distribution of grains is shown not to correlate with the calculated values (χ^2-test: Table 5.3 : last column). The distribution in the unstimulated cells was considered in Chapter 4 (4:II:6). In both the outer and inner secretory cells, the proportion of silver grains over the large osmiophilic vacuoles initially remains the same until day 4 when there is an increase followed by a considerable decrease at day 6 (Fig. 5.7). The small osmiophilic vacuoles, however, show an immediate loss of radiolabel on stimulation followed by a steady increase after day 2.

In the cell walls, the inner and outer secretory cells do not show such parallel patterns of radiolabel re-distribution. The silver grains over both wall regions of the outer cell layer initially increase and then are rapidly lost from the primary wall but more slowly lost from over the labyrinthine wall. In the inner secretory cells, however, there is little change in the primary wall content while the labyrinthine wall shows an initial large increase which does not dissipate as rapidly as in the outer secretory cells but does decrease slightly.

As with the unstimulated mature cells, the nucleus, mitochondria, plastids, lipid droplets and multivesicular bodies found in stimulated cells contain very little

radioactivity and, in general, the observed numbers of silver grains over these organelles are less than or equivalent to the calculated expected number. Very few silver grains overlie dictyosomes and these are the same as, or only slightly above, the expected numbers. In all these organelles, the numbers of grains show very little change as a result of stimulation (Table 5.3).

The endoplasmic reticulum in both secretory cell layers shows a considerable loss of radiolabel within two days of stimulation (Fig. 5.7). In the outer secretory cells, radiolabel then starts to reappear whereas in the inner secretory cells the loss continues until day 4, after which there is a rapid return of radiolabel to this compartment. The ground-plasm has an initial rapid doubling of its radio-label content which then shows little further alteration up to day 6 in either secretory cell layer.

III DISCUSSION

1) General Aspects of the Discharge of Secretion

The rate of release of fluid by Dionaea under conditions of regular daily stimulation is found to be approximately linear. Small fluctuations in the rate do occur, probably due to day-to-day changes in the prevailing environmental conditions. Even in the growth cabinets, where light and temperature are controlled, there are minor fluctuations in the level of water availability and this may have some effect on the volume of fluid released. The day-to-day variations in the volume of secretion are not great and normally any change is compensated for by an opposite change in the concentration of protein, leading to a very even profile for the secretion of protein (Fig. 5.4). After six to seven days, despite continued stimulation, the rate of discharge may decrease, indicating that the presence of nitrogenous stimulant is not the only factor involved in controlling the secretory cycle. If, however, the stimulant is removed, secretion ceases abruptly and the lobe re-opens within 48 h (Darwin, 1875).

If traps are stimulated to secrete under more natural conditions, such as by feeding a Blue-bottle fly (Calliphora sp.), then the rate of secretion per secreting lobe is again approximately linear (Fig. 5.1). When, however, the amount of secretion is recorded per calendar day for a whole plant, rather than as from the start of secretion for each lobe, then the volume and protein concentration of the secretion both increase to a peak after which the amount discharged

rapidly diminishes daily (Fig. 5.2). The peak of volume secretion is reached after seven days whereas the peak of protein is attained after only four days. The time-course for protein secretion corresponds closely to that of Scala et al (1969) who also recorded a peak of protein concentration after four days. They obtained a peak value of 22 mg ml^{-1} as against 2.8 mg ml^{-1} recorded here. Possibly their very high value was due to there being no precautions to prevent the fluid being contaminated with the 2%(w/v) gelatine which they used as a stimulant.

The bactopeptone preparation used as a stimulant for most of the experiments here contains a mixture of peptides, free amino acids, amides, ammonia and inorganic ions (Difco Laboratories, 1953), making it an ideal substitute for the natural hydrolytic digest which is produced during digestion. In addition, its low tryptophan content ensures that the background reaction with the determination of protein by the method of Lowry et al (1951) is negligible. Fig. 5.1 shows that the effect of stimulation with a 2%(w/v) bactopeptone solution is similar to stimulation by whole Calliphora sp. The amount of secretion produced is greater as this experiment was performed a year later than the others given in Fig. 5.1, by which time the lobes had grown considerably larger.

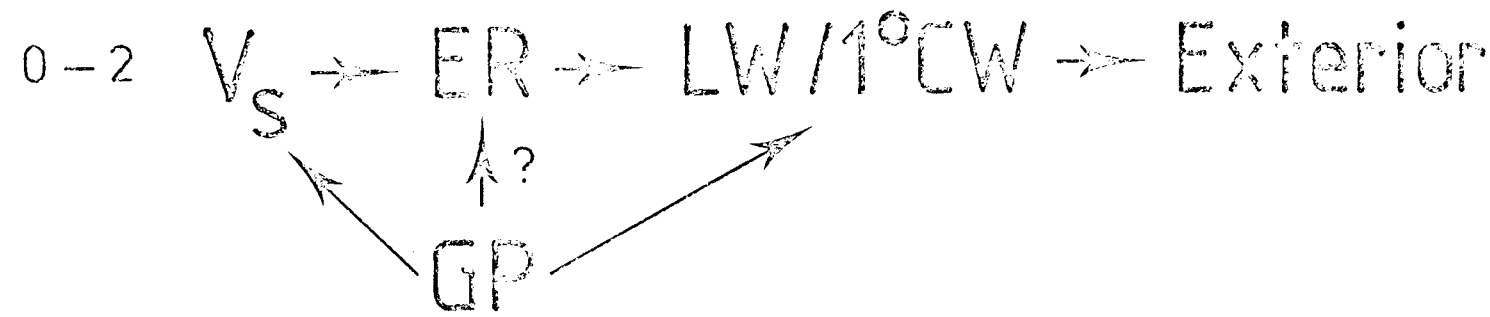
Thus the glands, which secrete only in response to a chemical stimulus, appear to maintain secretion only while stimulus continues to be administered. They do not, however, continue secreting until stimulation ceases but after six to eight days the amount of discharge may decrease, despite

no alteration in the load of stimulant being administered. This is presumably a natural mechanism to prevent an excessive discharge of hydrolases towards the end of the cycle when, despite the presence of small amounts of stimulant in the trap, it is uneconomical to continue the discharge of further hydrolytic enzymes (see Chapter 8).

2) Ultrastructural Aspects of the Discharge of Secretion

At least some of the ultrastructural changes reported in Chapter 3 are likely to be concerned with the discharge of secretion. Dynamic, stimulus-induced ultrastructural changes are shown to occur in all three vacuolar compartments, the cell walls, the endoplasmic reticulum and the ground-plasm. Of these, the osmiophilic vacuoles, the cell walls and the smooth endoplasmic reticulum are shown to contain stored protein while the polysomal rough endoplasmic reticulum is apparently synthesizing further secretory proteins. The autoradiographs (Table 5.3 and Fig. 5.7) show that a stimulus-induced redistribution of radiolabel occurs within these compartments. During the first two days following stimulation, the radiolabel which is lost from the small osmiophilic vacuoles and endoplasmic reticulum appears in the cell walls, a large proportion of it in the labyrinthine part of the wall, and the ground-plasm. Subsequently, there is a steady loss of radiolabel from the cell walls to the exterior. Secretion collected over these six days has a mean value of 800 counts min^{-1} ml^{-1}. By day 6, however, the grain count for the labyrinthine wall of the inner secretory cell is still considerably higher than prior to

DAYS


0 – 2
V_S
ER
LW/1°CW
Exterior
?
GP
2 – 4
V_S
ER
LW/1°CW
Exterior
4 – 6
V_S
ER
LW/1°CW
Exterior
?
V_L

stimulation, implying that discharge still continues up to and beyond this point.

After day 2 (outer secretory cells) and day 4 (inner secretory cells), the amount of radiolabel in the endoplasmic reticulum rises once more, suggesting that new radiolabelled protein is being synthesized. As there is a concurrent increase in grain-count for the small osmiophilic vacuoles, it appears that some of this newly-synthesized protein is being moved to that site. Alternatively, the counts in the small osmiophilic vacuoles and endoplasmic reticulum are derived from the depletion of the large osmiophilic vacuoles which occurs after day 4. No alteration is seen in the counts associated with dictyosomes or dictyosomal vesicles to indicate that this organelle is involved in the movement of proteins within the cells.

The flux pathway, proposed on the basis of these observations, is given opposite.

The endoplasmic reticulum plays a key role in the discharge of secretion. As discussed in Chapter 4, it probably serves as an important store of hydrolases as well as being the site of their synthesis. The way in which it is frequently stacked close to the plasmalemma suggests it has a direct role in the discharge and the observations of fusions of the reticulum membranes with the plasmalemma confirm this. In Pharbitis nil, Unzelman and Healey (1974) show that the endoplasmic reticulum synthesizes, stores and secretes the secretion. In Pharbitis, where the total secretion occurs within a few hours, numerous observations of fusion between the endoplasmic reticulum and plasmalemma

are seen simultaneously. In Allium quiescent root meristems, Bal and Payne (1972) show stacks of endoplasmic reticulum, some of which are fused to the plasmalemma, and suggest that these are discharging cell wall degrading enzymes directly into the apoplast.

By using cytochemical methods Vintéjoux (1973b, c) showed the secretion of Utricularia to contain both protein and polysaccharide and, later, that both dictyosomes and rough endoplasmic reticulum are involved (Vintéjoux, 1974a, b). In Drosophyllum Schnepf (1961a, b, c) shows that the secretion, which here only contains polysaccharide, originates at the dictyosomes and the vesicles from these move directly to the plasmalemma, while in the stalked glands of Drosera the mode of discharge is essentially similar except that the dictyosomal vesicles may fuse together, forming large secretory vacuoles which then fuse to the plasmalemma (Dexheimer, 1972; Schnepf, 1961a). There is autoradiographic evidence from root-caps (Dauwalder and Whaley, 1974; Juniper and Roberts, 1966; Kirby and Roberts, 1971; Northcote and Pickett-Heaps, 1966; Paull and Jones, 1975; 1976) and other polysaccharide secreting systems (Callow and Evans, 1974; Chrispeels, 1976; Ramus, 1972) and strong ultrastructural evidence from other plant glands (Schnepf, 1974; Schnepf and Busch, 1976) that in those species which secrete polysaccharide alone the dictyosomes are the sole site of origin of secretion. Normally discharge occurs by the direct fusion of dictyosomal vesicles to the plasmalemma. Although it is established that secretory proteins are synthesized at the rough endoplasmic reticulum (Lampen, 1974) there is

less evidence available for a discharge by direct fusion of endoplasmic reticulum to the plasmalemma.

In Mercurialis annua Figier (1969) found by auto-radiography that protein secreted from the petiolary gland is apparently synthesized at the rough endoplasmic reticulum and migrates to the dictyosomes. From here it does not pass directly to the plasmalemma but to smooth endoplasmic reticulum and membranous whorls, before finally being discharged. As the secretion contains both polysaccharide and protein (Figier, 1968b), he suggests that the dictyosomes are providing the polysaccharide component. Again in Pharbitis nil the secretion contains both protein and polysaccharide and both dictyosomes and rough endoplasmic reticulum are involved (Unzelman and Healey, 1974). The placentary papillae of the ovary in both Aptenia cordifolia and Platythyra haeckeliana also secrete a slime containing polysaccharide and protein and here too the dictyosomes and rough endoplasmic reticulum are involved (Kristen, 1976; 1977a). In the stigmatic papillae of A. cordifolia, however, only the rough endoplasmic reticulum appears to be involved even though the secretion contains polysaccharide as well as protein (Kristen, 1977b). In this gland, however, the endoplasmic reticulum swells to form vesicles which are then discharged. But in the leaf-glands of Nomphalia stricta the proteinaceous secretion is apparently released directly from the rough endoplasmic reticulum (Kristen, 1975).

It appears, therefore, that, in general, when the secretion contains both polysaccharide and protein then both dictyosomes and rough endoplasmic reticulum are involved:

when just polysaccharide is present then only the dictyosomes are involved: and when protein alone is secreted then only the endoplasmic reticulum is involved. Dionaea falls into the last of these categories. Whether these fusions between the endoplasmic reticulum and the plasmalemma are a dynamic or a static process in Dionaea cannot be deduced from electron micrographs, but the comparative scarcity of clear examples indicates that the process is probably dynamic.

Some profiles of endoplasmic reticulum are closely juxtaposed to the wall for several μm and have an asymmetrical distribution of ribosomes. These too are almost certainly concerned with the discharge of secretion. Similar profiles occur in developing phloem of Triticum (Pickett-Heaps and Northcote, 1966) where it is suggested that they are secreting callose precursors, as they lie over the points where sieve-pores develop. A more likely possibility, however, is that they are secreting the synthetase enzymes required to make callose and perhaps some of the hydrolases which degrade the wall during the formation of the pore.

Dictyosomes do not appear to play any role in the secretion of hydrolase activity in Dionaea. Not only is there apparently a decrease in the total number of dictyosomes but no significant stimulus-induced change occurs in either their structure or the number of associated vesicles (Fig. 3.44). Dictyosomal vesicles are rarely seen fusing to the plasmalemma and where this does occur it does not appear to be involved with the secretion of hydrolases (see 3:IV:3). Furthermore, there are very few silver grains associated with the dictyosomes and these show no stimulation-induced

fluctuation (Table 5.3).

3) The Effects of Colchicine, Cytochalasin B, and Cycloheximide

When traps are stimulated in the presence of colchicine there is no effect on the volume of fluid produced nor on its protein content, yet the enzymatic activity is greatly decreased. Cytochalasin B has a very similar effect, although these two drugs do not always show the same effect against a particular system. For example, with human leucocytes colchicine inhibits the release of lysosomal hydrolases whereas cytochalasin B increases it (Allison and Davies, 1974).

The observed effects of colchicine on Dionaea secretory activity are not compatible with the inhibitory action of this drug being due to the disruption of microtubules, as may be the case in a number of other stimulus-induced secretory systems (Allison and Davies, 1974). If the action of colchicine was directed against microtubules which were organising the passage of packages of hydrolytic enzymes to the cell periphery then the decrease in enzyme activity should be matched by a decrease in the total protein secreted. That this is not the case is clearly shown in Table 5.1. Where colchicine has been seen to disrupt secretion, either inhibiting the discharge of secretory product (Allison and Davies, 1974) or upsetting the organisation of the discharge (Pickett-Heaps, 1967), then the mechanism of secretion usually involves a packaging system of Golgi-derived vesicles. It has already been shown that dictyosomes appear

not to play any role in the release of hydrolase activity in Dionaea and the effect of colchicine supports this. As, however, tissue was normally fixed in the cold microtubules would not have been preserved and therefore it has not been determined whether they are involved at all in the secretory process.

Hence colchicine is apparently acting elsewhere but the action is not directed against the enzymes themselves (Table 5.2). Stadler and Franke (1972; 1974) have shown colchicine to bind non-specifically to proteins in cell membranes and that up to 50% of the colchicine may be membrane-bound in liver and brain tissue. They show the binding to be stable, to increase with temperature, to be unaffected by lipid-depletion, and to vary from one membrane type to another. Furthermore, this binding shows different binding kinetics and thermostability properties from the binding with tubulin, implying that it is not due to artefactual tubulin contamination of the membranes (Stadler and Franke, 1974). Working with Tetrahymena pyriformis, Wunderlich et al (1973) have shown colchicine to cause clumping of those granules in the plasma membrane which Satir (1974) has elegantly demonstrated to be a vital component in the fusion of mucocysts with the plasma membrane. Here then is a clear example where colchicine disrupts secretion by making the fusion event of discharge more difficult.

Although there is no evidence for such a fusion mechanism occurring in Dionaea, colchicine could be acting by preventing the endoplasmic reticulum from fusing to the

plasma membrane as readily as under normal conditions. If this were the only effect, however, then an overall decrease in the protein content of the secretion would still be expected.

Another important effect of colchicine is that it decreases the synthesis of ribonucleic acids and proteins (Creasey and Markiw, 1964) and deoxyribonucleic acid (Jones et al, 1966). There is again no evidence that colchicine is having any of these effects in Dionaea. It is, however, worth noting that in Figs. 5.5 and 5.6 the rates of secretion of acid protease and acid phosphatase in colchicine-treated samples are greater over the first few days of secretion after which they diminish, while the control maintains a constant rate for several further days. This is compatible with an effect of the drug on the synthesis of these enzymes upon the cisternae of endoplasmic reticulum.

What, then, may be happening is that the drug is interfering with the synthesis of hydrolases intended for secretion and causing incomplete or incorrectly formed proteins to be released. Certainly vinblastine, a drug closely related to colchicine, has been shown to induce a peculiar appearance in the endoplasmic reticulum of Earle's L-922 mouse fibroblasts with stacks of regularly constricted cisternae forming near the nucleus (Krishan et al, 1968). In addition, the drug may be preventing proper fusion of the endoplasmic reticulum to the plasmalemma and delaying the release of the enzymes. If, as a result of this delay, the hydrolases became active then partial autolysis prior to secretion would explain the observed effects. Much of this

is speculative and further work is required to establish the cause of the observed activity of colchicine against this system.

Although cytochalasin B has very similar effects to colchicine on the quality of the secretion released it does not a priori follow that the mechanism by which this effect is caused will be the same. It is generally accepted that cytochalasin B binds to microfilaments and many of the effects of this drug, notably on cytoplasmic streaming and cell motility are due to this action (Wessels et al, 1971), as may be some of its effects on secretion (Allison and Davies, 1974). Thus in maize root-tip cells the migration of dictyosomal vesicles to the plasmalemma is inhibited by cytochalasin B, but not their formation (Mollenhauer and Morre, 1976). Similarly, in the development of Microsterias the synthesis of cell-wall precursors is severely upset by cytochalasin B (Tippit and Pickett-Heaps, 1974). In neither of these cases does colchicine show much effect.

As with colchicine, however, cytochalasin B also appears to have a direct effect on membrane transfer processes. Thus it inhibits the uptake of 2-deoxy-D-glucose by fibroblasts (Kletzein et al, 1972), 2-deoxy-D-glucose and D-glucosamine by leucocytes (Zigmond and Hirsch, 1972), sucrose by liver cells (Wagner et al, 1971) and nucleosides by hepatoma cells (Plagemann and Estensen, 1972) but the same concentrations of drug do not inhibit the uptake of L-leucine by fibroblasts and leucocytes (Zigmond and Hirsch, 1972) or choline by hepatoma cells (Plagemann and Estensen, 1972). Of these only the inhibition of sucrose uptake may

be readily explained as an action against microfilaments. Furthermore, Sanger and Holtzer (1972) have found it to cause a decrease in the synthesis of cell-surface glycoproteins but in the other systems, mentioned above, where a decrease in synthesis was observed this was attributed to an inhibition at the level of membrane transport, and thus a depletion of the precursor pool. Zigmond and Hirsch (1972) observed a 70 - 90% inhibition of glycoysis in whole cells which was eliminated when the cells were ruptured.

Hence it seems possible that cytochalasin B is also having an effect on the discharge of secretion by disrupting the events at the plasmalemma rather than within the cytoplasm. Further work, including binding studies with radiolabelled colchicine and cytochalasin B, is needed to elucidate the details of how this activity is being expressed.

The release of α-amylase from barley aleurone tissue was found to be unaffected by cycloheximide or actinomycin D by Varner and Mense (1972), but Vigil and Ruddat (1973) found a 63% inhibition of the discharge of this enzyme by actinomycin D while there was only a 45% inhibition of its synthesis. Furthermore they found during the germination of these seeds that there was an extremely active rough endoplasmic reticulum with numerous associated polysomes. Secretion appeared to be directly from the rough endoplasmic reticulum by small smooth vesicles and actinomycin D was seen to impair the formation of these vesicles. Possibly the action of cycloheximide in Dionaea is similar, as a considerable effect on the discharge of the enzymes was seen without any change in the amount of protein released, while the decrease in

radiolabel incorporation into the secretory proteins was only about 30%. Vigil and Ruddat (1973) suggest that aetinomycin D may be affecting the formation of endoplasmic reticulum. In contrast to these effects on the release of α-amylase Morre et al (1967) found puromycin not to affect the discharge of root-cap slime. This is compatible with the important distinctions apparent between systems secreting proteins and those secreting polysaccharides. Both the release of α-amylase (Varner and Mense, 1972) and of root-cap slime (Morre et al, 1967) are, however, strongly energy-dependent.

Possibly, therefore, the effect of all these drugs in Dionaea is due to a disruption of complete protein synthesis on the membranes of the endoplasmic reticulum. The observed effect of colchicine and cytochalasin B on the membranes of other tissues supports this possibility. The large mitochondrial population of Dionaea secretory cells and the stimulus-induced changes observed within them (see 3:IV:3) strongly suggest that secretion by Dionaea is also energy-dependent, but no experiments have been done to confirm this. A further possibility is that these drugs are preventing the synthesis or expression of an activating system for the secretory hydrolases. Possibly, as in the pancreas, they are made in an inactive form requiring selective proteolysis to become active. Drugs which prevented this activation without affecting the secretory activity would cause secretion to be released containing normal amounts of protein but diminished quantities of hydrolytic activity, as observed here.

Table 5.1: The Effect of the Drugs Colchicine and Cytochalasin B on the Composition of the Secretion: Cumulative Mean % of the Control after Six Days Stimulation

22 lobes were used for each treatment, and for the control, in three separate experiments

	Colchicine*	Cytochalasin B**
Fluid volume ($\mu l\ 100\ mm^{-2}$)	112.2±15.4	119.3±7.2
Protein ($\mu g\ 100\ mm^{-2}$)	110.7±10.6	127.4±1.2
Protein concentration ($mg\ ml^{-1}$)	101.3±3.3	107.1±5.4
Protease activity ($pkat(mg\ protein^{-1})$)	43.3±1.7	52.3±1.6
Protease activity ($pkat(mg\ protein^{-1})mm^{-2}$)	43.0±8.1	62.3±9.3
Acid phosphatase activity ($\mu kat(mg\ protein^{-1})$)	65.5±14.9	49.9±10.7
Acid phosphatase activity ($\mu kat(mg\ protein^{-1})mm^{-2}$)	76.1±7.9	69.0±13.7

*10^{-3}M and 10^{-4}M colchicine in 4%(w/v) bactopeptone solution.

**5$\mu g\ ml^{-1}$ and 10 $\mu g\ ml^{-1}$ cytochalasin B in 4%(w/v) bactopeptone solution containing 1%(v/v) DMSO.

Table 5.2: Effect of the Drugs Colchicine and Cytochalasin on Enzymatic Activity of the Secretion

20 µl of crude secretion was incubated in 1.0 ml of buffered solution containing the given concentration of drug.

Amount of Drug Present		Enzyme Activity*	
Colchicine (µmol)	Cytochalasin B (µg)	Acid Protease (% of control)	Acid Phosphatase (% of control)
0.01	-	137.8	94.2
0.02	-	137.8	97.4
0.04	-	129.7	101.3
0.10	-	151.4	96.8
0.20	-	102.7	92.3
-	0.1	140.5	100.6
-	0.2	132.4	96.2
-	0.5	113.5	97.4
-	1.0	164.9	107.7
-	2.0	140.5	103.9

*Control values: protease 2.9 nkat (ml secretion)$^{-1}$
acid phosphatase 5.1 nkat (ml secretion)$^{-1}$

Table 5.3: The Redistribution of Silver Grains within the Gland Secretory Cells following Stimulation

A) Outer Secretory Cells

Days After Stimulation	Silver Grain Distribution	Cytoplasmic Compartment															χ^2-test
		1°CW	LW	V_C	V_1	V_S	MVB	N	M	LD	P	D	DV	ER	GP	Total	
0	Observed % grains present	14.1	9.5	-	25.5	17.1	0	1.5	3.4	0	0.8	2.3	1.9	14.8	8.4	-	1690.27
	Observed total grains present	38	25	-	67	45	0	5	9	0	2	6	5	39	22	263	p<0.00.
	Calculated total grains present	32	5	-	109	12	<1	26	10	0	5	4	1	1	58	263	
2	Observed % grains present	21.1	17.9	4.2	24.2	2.1	0	2.1	3.2	0	0	2.1	1.1	4.2	17.9	-	92.36
	Observed total grains present	20	17	4	23	2	0	2	3	0	0	2	1	4	17	95	p<0.001
	Calculated total grains present	13	3	8	21	4	0	7	9	<1	1	1	1	16	11	95	
4	Observed % grains present	9.3	16.5	5.2	33	5.2	0	2.1	1.0	0	0	0	2.1	10.3	15.5	-	57.24
	Observed total grains present	9	16	5	32	5	0	2	1	0	0	0	2	10	15	97	p<0.001
	Calculated total grains present	13	4	7	20	6	<1	6	7	<1	3	1	1	11	18	97	
6	Observed % grains present	9.3	10.8	11.2	13.4	9.3	0	1.9	8.2	0	0.4	1.9	3.4	11.2	19.0	-	116.15
	Observed total grains present	25	29	30	36	25	0	5	22	0	1	5	9	30	51	268	p<0.001
	Calculated total grains present	32	7	30	76	17	0	27	13	0	5	2	2	20	37	268	

Table 5.3 cont'd

B) Inner Secretory Cells

Days After Stimulation	Silver Grain Distribution	Cytoplasmic Compartment														χ^2-test
		1°CW	LW	V_C	V_1	V_S	MVB	N	M	LD	P	D	DV	ER	GP	Total	
0	Observed % grains present	8.1	9.4	-	20.8	18.8	0	6.7	8.7	0	0	0	2.0	16.8	8.7	-	86.43
	Observed total grains present	12	14	-	31	28	0	10	13	0	0	0	3	24	13	149	p<0.001
	Calculated total grains present	13	5	-	23	24	0	19	10	<1	3	<1	<1	7	46	149	
2	Observed % grains present	6.0	28.0	14.0	20.0	0	0	0	2.0	0	0	0	2.0	8.0	20.0	-	46.67
	Observed total grains present	3	14	7	10	0	0	0	1	0	0	0	1	4	10	50	p<0.001
	Calculated total grains present	6	4	4	16	1	0	4	3	0	2	0	0	6	4	50	
4	Observed % grains present	10.4	16.7	4.2	33.3	6.3	0	2.1	6.3	0	0	0	2.1	2.1	16.7	-	19.06
	Observed total grains present	5	8	2	16	3	0	1	3	0	0	0	1	1	8	48	p<0.05
	Calculated total grains present	3	4	2	11	3	<1	7	3	0	1	1	<1	6	7	48	
6	Observed % grains present	6.3	22.2	4.9	8.3	9.7	0	1.4	6.9	0	0.7	0	4.2	13.9	21.6	-	-
	Observed total grains present	9	32	7	12	14	0	2	10	0	1	0	6	20	31	144	

Figure 5.1 Cumulative time-course of the volume of secretion produced in response to different stimuli: plotted per secreting lobe as of the first day after each lobe started secreting. The volume of stimulant solution administered each day varied from 50 - 200 µl.

Symbol	Stimulant Applied	No. Traps
●	2% (w/v) bactopeptone	15
○	live Calliphora sp. (approx. 60 mg) sealed in a dialysis membrane	8
▲	6.8 mM-L-glutamine	11
△	16.7 mM-urea	11
■	6.0 mM-uric acid	7
□	58.7 mM-ammonia	7
◆	6.1 mM-L-phenylalanine	8

All these results, except those for the 2% (w/v) bacto-peptone solution, are re-plotted from Robins (1975; 1976).

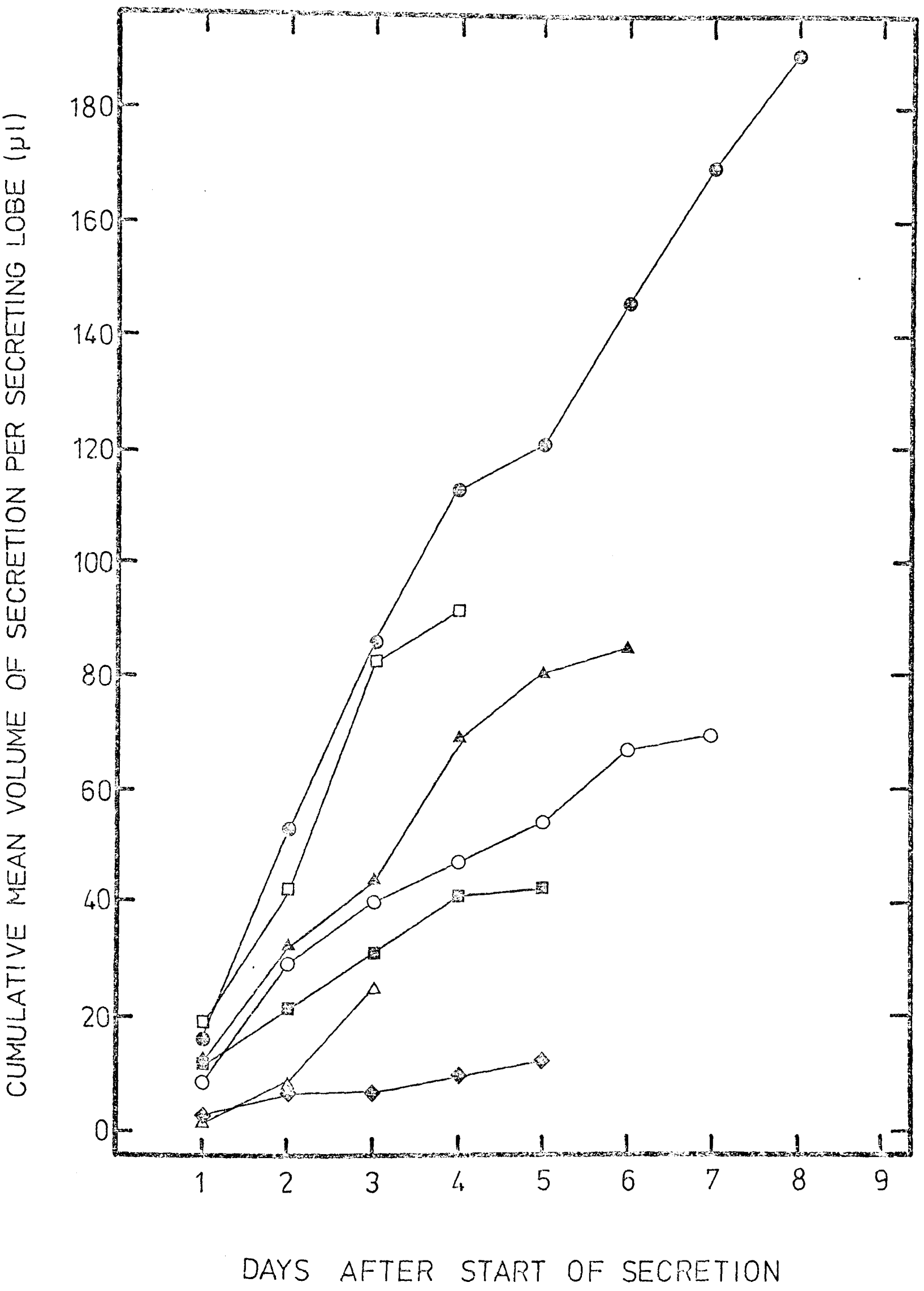

CUMULATIVE MEAN VOLUME OF SECRETION PER SECRETING LOBE (µl)
0
20
40
60
80
100
120
140
160
180
1
2
3
4
5
6
7
8
9
DAYS AFTER START OF SECRETION

Figure 5.2 Time-course of secretion by traps fed with natural prey: plotted per secreting lobe as from the day on which prey was supplied.

a) Mean volume of fluid per secreting lobe from plants in which the traps were each supplied with a live Calliphora sp.;

b) Mean Concentration of protein per secreting lobe from plants in which the traps were each supplied with a live Calliphora sp. sealed into a dialysis membrane.

Number of traps per experiment:

a) 28

b) 8

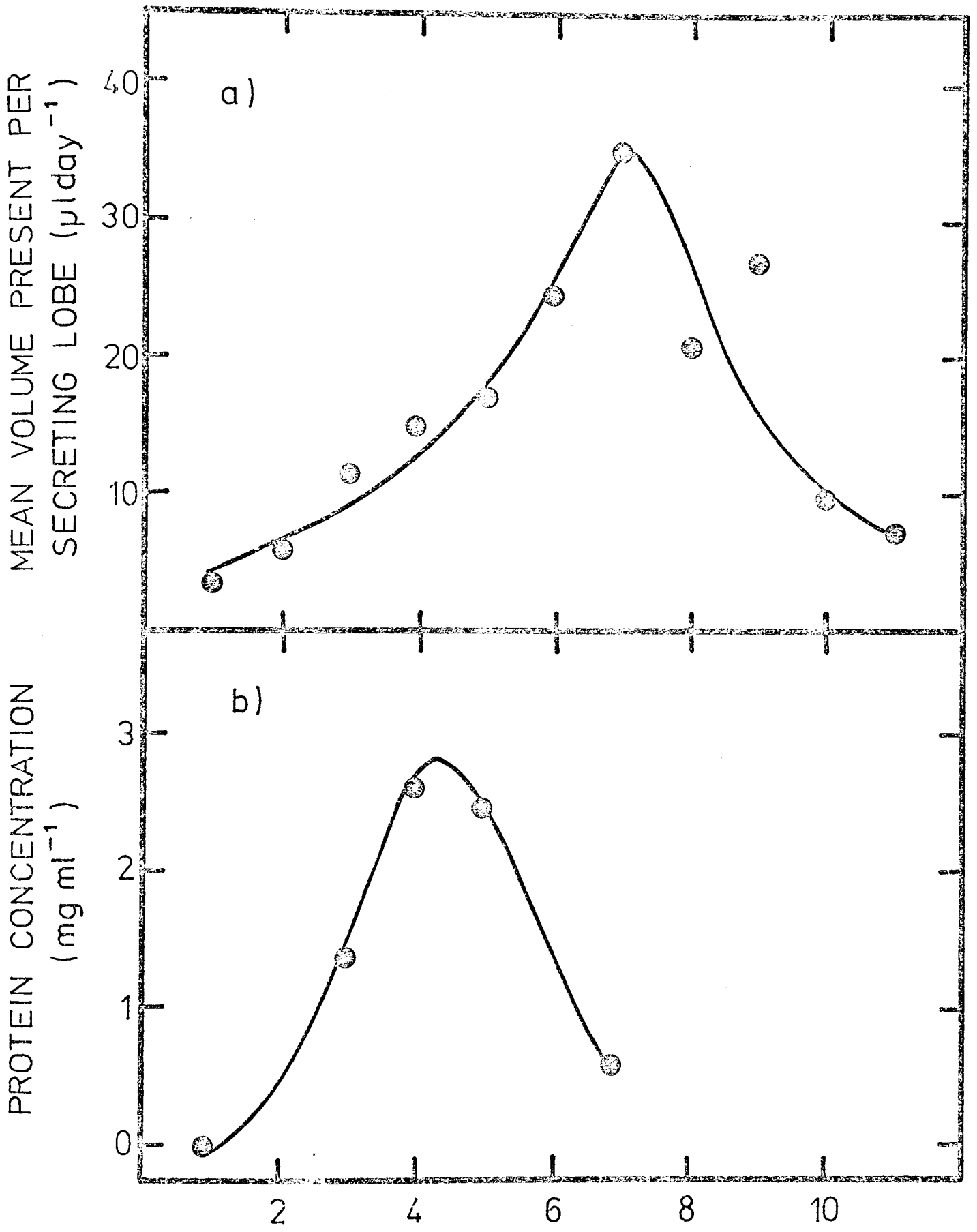

a)
b)
MEAN VOLUME PRESENT PER SECRETING LOBE (µl day⁻¹)
PROTEIN CONCENTRATION (mg ml⁻¹)
40
30
20
10
3
2
1
0
2
4
6
8
10
DAYS AFTER FEEDING

Figure 5.3 Cumulative time course of the mean volume of secretion produced in the presence of different drugs: plotted per secreting lobe as of the first day after each lobe started secreting.

Symbol	Stimulant Applied	No. Traps	Mean Area (mm^2)
●	4%(w/v) bactopeptone	9	208±29
■	10^{-3}M-colchicine in 4%(w/v) bactopeptone	9	201±33
▲	10 µg ml^{-1} cytochalasin B in 4%(w/v) bactopeptone	9	205±23

Figure 5.4 Cumulative time course for protein secretion in the presence of different drugs: plotted per secreting lobe as of the first day after each lobe started secreting.

a) Cumulative mean protein content per unit area;

b) Cumulative mean protein concentration.

Symbol	Stimulant Applied	No. Traps	Mean Area (mm^2)
●	4%(w/v) bactopeptone	8	249±27
■	10^{-3}M-colchicine in 4%(w/v) bactopeptone	8	319±40
▲	10 µg ml^{-1} cytochalasin B in 4%(w/v) bactopeptone	8	283±37

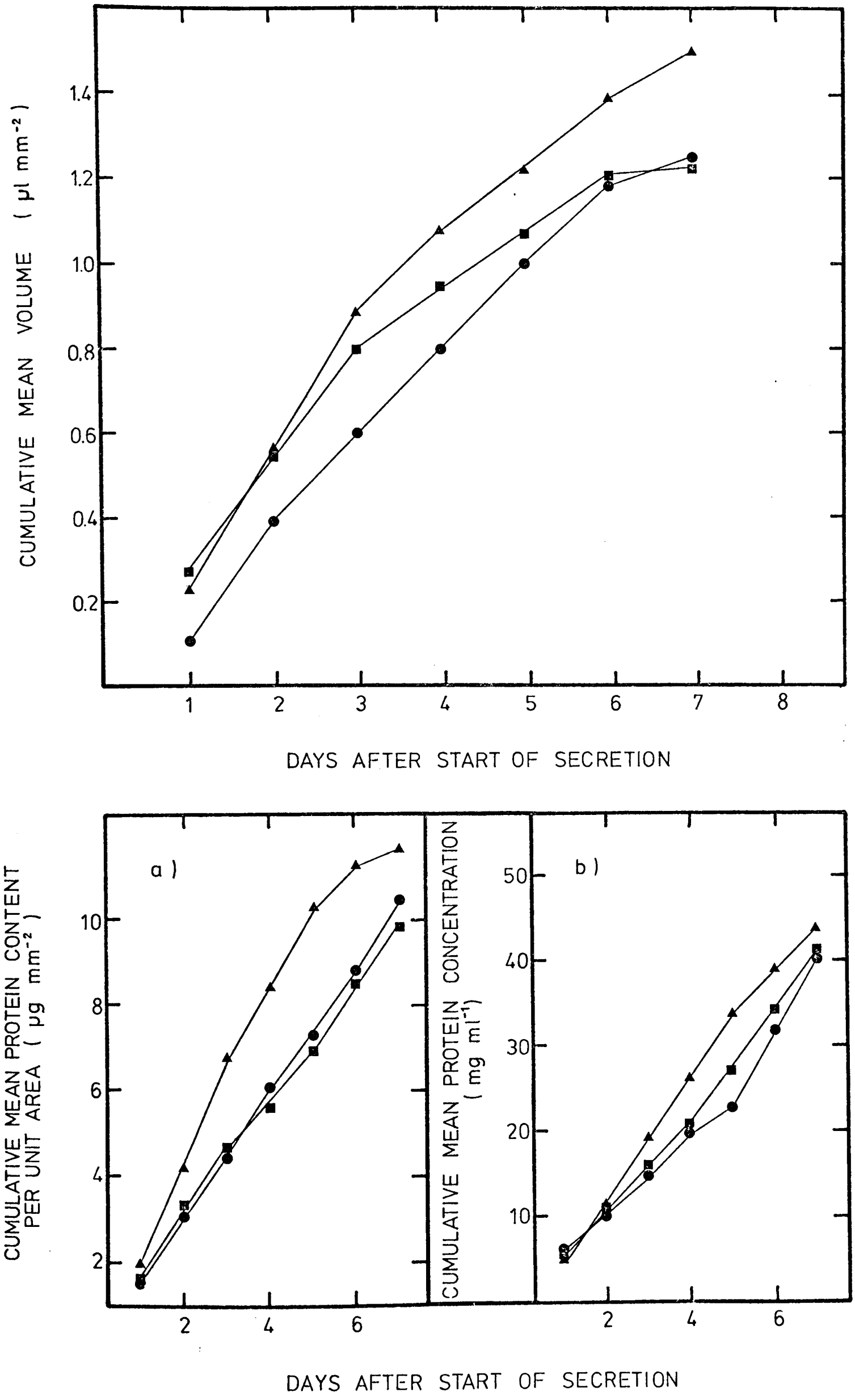

CUMULATIVE MEAN VOLUME (µl mm⁻²)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
1
2
3
4
5
6
7
8
DAYS AFTER START OF SECRETION
CUMULATIVE MEAN PROTEIN CONTENT PER UNIT AREA (µg mm⁻²)
a)
10
8
6
4
2
2
4
6
CUMULATIVE MEAN PROTEIN CONCENTRATION (mg ml⁻¹)
b)
50
40
30
20
10
2
4
6
DAYS AFTER START OF SECRETION

Figure 5.5 Cumulative time course of the release of hydrolase activity in the presence of different drugs: plotted per secreting lobe as of the first day after each lobe started secreting.

a) Cumulative mean specific acid protease activity;

b) Cumulative mean specific acid phosphatase activity.

Details as in Fig. 5.4.

Figure 5.6 Cumulative time course of the release of hydrolase activity per unit area in the presence of different drugs: plotted per secreting lobe as of the first day after each lobe started secreting.

a) Cumulative mean specific acid protease activity per unit area;

b) Cumulative mean specific acid phosphatase activity per unit area.

Details as in Fig. 5.4.

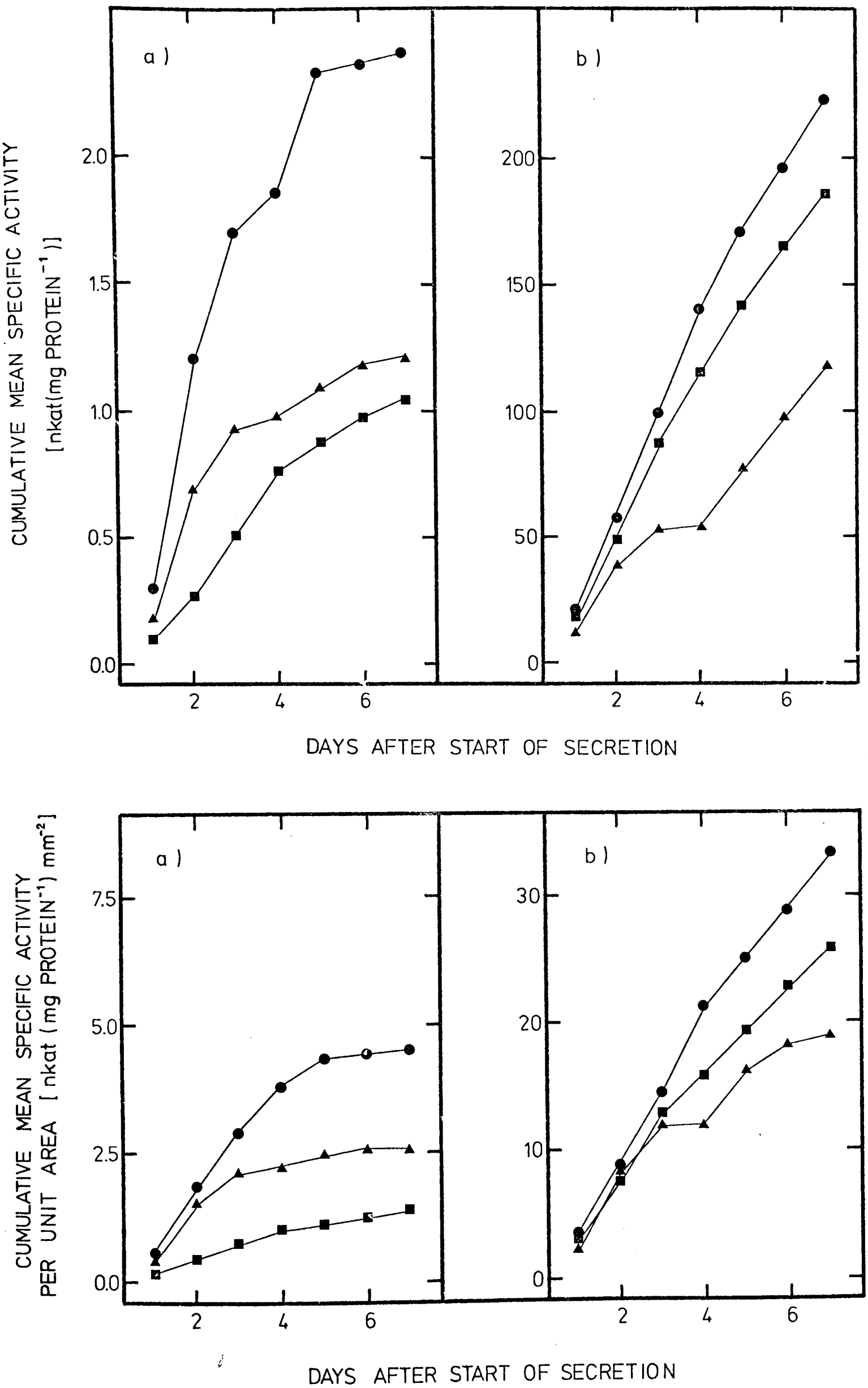

a)
b)
CUMULATIVE MEAN SPECIFIC ACTIVITY
[nkat(mg PROTEIN^{-1})]
2.0
1.5
1.0
0.5
0.0
200
150
100
50
0
2
4
6
DAYS AFTER START OF SECRETION
CUMULATIVE MEAN SPECIFIC ACTIVITY
PER UNIT AREA [nkat (mg PROTEIN^{-1}) mm^{-2}]
7.5
5.0
2.5
0.0
30
20
10
0
2
4
6
DAYS AFTER START OF SECRETION

Figure 5.7 Showing the dynamic re-distribution of silver grains within different sub-cellular compartments of the secretory cells following stimulation with 4%(w/v) bacto-peptone solution. Plotted from Table 5.3.

a) Large osmiophilic vacuole;

b) Small osmiophilic vacuole;

c) Primary cell wall;

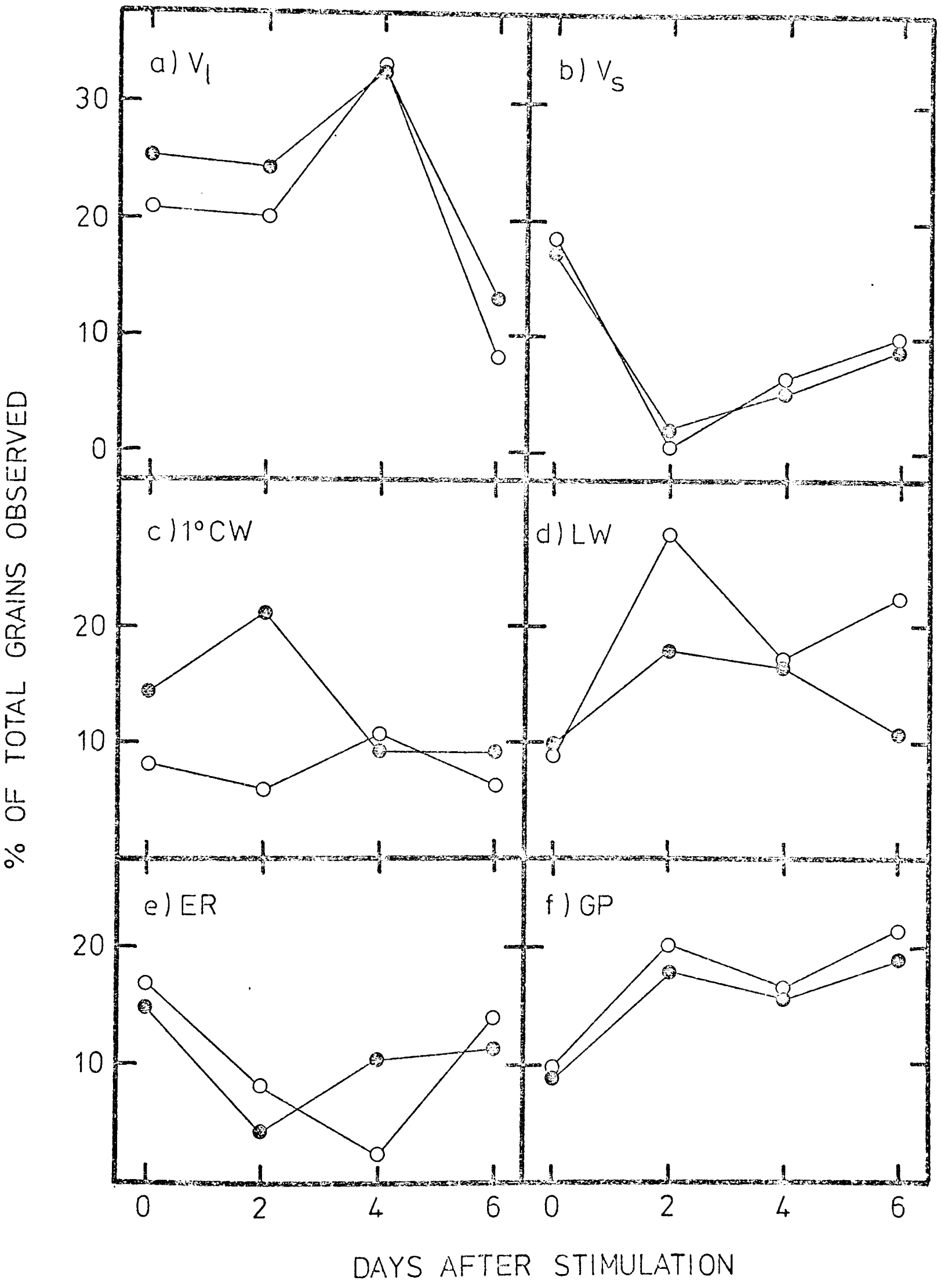
d) Labyrinthine cell wall;

e) Endoplasmic reticulum;

f) Ground-plasm.

Outer secretory cell ●

Inner secretory cell ○


a) V_l
b) V_s
c) 1°CW
d) LW
e) ER
f) GP
30
20
10
0
20
10
20
10
0
2
4
6
0
2
4
6
% OF TOTAL GRAINS OBSERVED
DAYS AFTER STIMULATION

CHAPTER 6

ENZYMOLOGY OF THE SECRETION

I INTRODUCTION

II RESULTS

1) Peptide Hydrolase Activity

2) Acid Phosphatase Activity

3) Chitinase Activity

4) Peroxidase Activity

III DISCUSSION

1) Peptide Hydrolase Activity

2) Phosphatase Activity

3) Chitinase Activity

4) Peroxidase Activity

5) Conclusions

I INTRODUCTION

The enzymology of the secretion from Dionaea has received very little attention. In the last century, Balfour (1875) and Darwin (1875) both demonstrated the digestive ability of the fluid but since then there have been only two further contributions. Lüttge (1964a) showed that the secretion resembles that of Nepenthes in containing proteinase activity and several peptide hydrolases (Table 6.1). A few years later, Scala et al (1969) confirmed the presence of a proteinase but claimed that "proteinase activity could be detected only when a large protein was used as substrate". They also tested the secretion for some esterase (EC.3.1.3.), glycosidase (EC.3.2.1.) and anhydrase (EC.3.6.1.) activities and found several activities present within these groups (Table 6.1).

As pointed out by Scala et al (1969), the quantity of secretion produced by Dionaea is not large (approx. 100 µl $lobe^{-1}$ day^{-1}) and it was not found practical as part of the present investigation to purify extensively any of the enzymes of the secretion. So far, enzymatic activity against 13 different substrates has been shown for the crude secretion, while two more activities may be present (Table 6.1). A number of these are reported here for the first time.

II RESULTS

1) Peptide Hydrolase Activity

1. pH-dependence. When assayed using casein as substrate the pH-activity curve for Dionaea crude secretion shows two peaks, one at pH4.0 and the other at pH5.0 (Fig. 6.1a). Both peaks show a temperature-dependence although the pH4.0 peak is relatively more active at 25°C while the greater activity at pH5.0 occurs at 30°C. Assays were normally conducted at pH5.0 with an incubation temperature of 30°C.

2. Inhibition studies. The effect of incubating crude secretion in the presence of various known inhibitors specific to different peptide hydrolase activities is given in Table 6.2. Their effects on pepsin and papain incubated simultaneously are also given. Pepstatin and PMSF show very little inhibition of the peptide hydrolase activity of Dionaea secretion once corrected for the effect of the non-aqueous solvent present. Indeed the inhibition of pepstatin plus ethanol is less than ethanol alone at the same concentration. EDTA, iodoacetamide and acetic anhydride all inhibit the activity to some extent. With EDTA, 67.6% inhibition is observed at a concentration of EDTA at which papain is enormously stimulated. Iodoacetamide causes an equivalent degree of inhibition with secretion (21.6%) as it does with papain (24.6%) while acetic anhydride causes a 43.2% inhibition with secretion and apparently increases the activities of pepsin and papain.

3. Zymograms. Figs. 6.2b and 6.2c show typical zymograms

obtained by the electrophoresis of crude secretion in a gel pre-loaded with casein and treated as described in 2:IV:2. There are two major bands, A and D, which are rather broad and two narrow minor bands, B and C, between them. When electrophoresis is conducted in the presence of SDS then four bands are clearly visible. The banding pattern is not the same due to the altered conditions of electrophoresis (see Fig. 6.2 legend for details).

4. Substrate studies. To investigate further the peptide hydrolase activity of the secretion, it was tested against a number of substrates which are specific to particular types of proteolytic attack. The results of this are given in Table 6.3. The secretion shows reactivity at pH5.2 with N-CBZ-L-alanine-p-NPE, L-Leucyl-β-naphthylamide and N-CBZ-L--phenylalanine-p-NPE. A very slight reaction with BAME occurs at pH6.75 but this is unlikely to be physiologically important as no reaction occurs with this substrate at pH5.2. There is no detectable hydrolase activity against TAME or N-Benzoylglycyl-L-arginine.

2) Acid Phosphatase Activity

Acid phosphatase is confirmed as an important component of the hydrolase activity of the secretion. The pH-activity curve is found to be smooth with maximum activity at pH5.0 (Fig. 6.1b).

When secretion is subjected to PAGE and then stained for p-nitrophenyl phosphatase activity, a broad yellow band of mean mobility 0.48 (range ±0.04) develops (Fig. 6.2d). When

the gel is sliced this band shows a double peak of activity and there is a further band of activity at mobility 0.13, which is not apparent on the histochemical stain (Fig. 6.2e). Under isoelectric focussing, a single major peak of p-Nitrophenyl phosphatase activity at pI4.95 is obtained with a shoulder at pI3.80 (Fig. 4.37). Hence it would appear that there are at least two phosphatase activities against p-Nitrophenyl phosphate present, one of which is considerably more active than the other.

3) Chitinase

Fig. 6.3 shows the activity of secretion from Dionaea against colloidal chitin and compares this with the activity of a commercial preparation (SCC : from Streptomyces griseus). The rate of hydrolysis over the first 24 h is 1.64 pkat (mg protein)$^{-1}$ which, although low, is significant. The SCC preparation shows an initial rate at pH5.0 of 5.07 pkat (mg protein)$^{-1}$, only about x 3 the activity of Dionaea secretion. This is sufficient to cause an almost total depletion of the substrate (150 mg chitin ml^{-1}) present within 48 h, as observed by the total loss of turbidity in these vials.

4) Peroxidase

Secretion shows a concentration-dependent and pH-dependent peroxidase activity (Fig. 6.4). This enzyme must therefore be considered a component of the hydrolytic

activity of the secretion. The concentration-dependence of horseradish peroxidase (SCC) is non-linear over the range tested (Fig. 2.6) but with secretion the rate of reaction shows a linear relationship to the amount of protein present. The activity at pH5.0 is considerably greater than that at pH7.0, with mean specific activities of 0.86 and 0.46 nkat(mg protein)$^{-1}$ respectively. This is x 10^4 lower than the commercial preparation, where the mean specific activity is 8.6 μkat(mg protein)$^{-1}$, but the pH-dependence and concentration-dependence indicate that it is a real effect.

III DISCUSSION

PAGE of the crude secretion produces only four bands of protein (Fig. 6.2a) while four are also obtained by isoelectric focussing (Fig. 4.37). Thus, although there may be a few minor proteins which are not detected by these methods, it seems unlikely that all the activities listed in Table 6.1 are due to separate proteins. Instead, there are probably several enzymes present each of which has a very broad specificity. Thus, for example, some of the esterase activity may be due to broad-action peptide hydrolases. Further work is needed, however, to clarify the situation and the results presented here must be considered as only a very preliminary investigation.

1) Peptide Hydrolase Activity

The general proteolytic activity was assayed using casein for substrate as Lüttge (1964a) had found this to be readily hydrolysed by Dionaea secretion. Scala et al (1969) used a dye-fibrin complex method (Nelson et al, 1961) to assay for general protease but no activity could be obtained in the present study against this type of substrate (Azocoll : Calbiochem, La Jolla, USA) even after several hours incubation under a variety of conditions.

That more than one protease active against casein is present is shown by the zymograms produced by PAGE. These display two major bands of activity, while the two minor bands running between them (Fig. 6.2b) may be either minor

proteases or due to partial autolysis of the lower mobility band (A). When electrophoresis is conducted in the presence of SDS, however, then four rather sharper bands are obtained (Fig. 6.2c) suggesting that there are four separate proteases present. It is surprising that these enzymes remain active after treatment with SDS but the activities of both protease and acid phosphatase are readily detected after subjecting secretion to PAGE in the presence of SDS.

The pH-activity profile of crude secretion is not at all uniform (Fig. 6.1a) and is most readily interpreted as being due to a mixture of at least two protease activities, thus supporting the electrophoresis results. Scala et al (1969), however, found the pH-activity profile of the secretion to have a single very sharp peak at pH5.5. It appears, therefore, that with the dye-fibrin complex method they were detecting only one of the peptide hydrolases, presumably an endopeptidase.

Both Lüttge (1964a) and Scala et al (1969) have made some attempt to specify the types of proteolytic activity present. Lüttge (1964a) detected L-leucine aminopeptidase, glycylglycine dipeptidase, glycyl-L-leucine dipeptidase and possibly proline dipeptidase in the crude secretion. Scala et al (1969) could only detect proteolytic activity, however, when a large protein was used as substrate but do not report which small molecular substrates were tried. They were also unable to obtain tryptic or chymotryptic activities, using the methods of Hummel (1959) and Schwert and Takenaka (1955) respectively. On the basis of these results they concluded that "... the digestive fluid of Venus's-

flytrap contains a proteolytic enzyme ... it appears that the enzyme does not exhibit peptidase or esterase activity ... in most respects it is probably similar to papain" (Scala et al, 1969). In making this observation, they appear to have missed or ignored the work of Lüttge (1964a) while they give no justification for stating that the proteolytic activity is probably similar to papain.

No activity against BAME is present at pH5.2 (Table 6.3) but with 0.5 mM-iodoacetamide the activity in the crude secretion is inhibited to the same extent as is papain incubated simultaneously, suggesting that alkylation has an effect on one or more of the enzymes present. Thus, while one of the proteases present may be a thiol proteinase (EC.3.4.22.) it does not appear to have the same substrate specificity as papain (EC3.4.22.2) or stem bromelain (EC.3.4.22.4). A trace of activity is detected against BAME at pH6.75 but it seems unlikely that this is physiologically significant.

The results of this study confirm those of Scala et al (1969) that there is no tryptic activity (EC.3.4.21.4) in the secretion. A trace of activity against N-CBZ-L-phenylalanine-p-NPE was detected, but in view of the virtual lack of inhibition of activity by PMSF it seems likely that this may be due to the carboxypeptidase A (EC.3.4.12.2) present rather than to chymotrypsin (EC.3.4.21.1).

Carboxypeptidase A is the only peptide hydrolase clearly shown to be present in the secretion in this study but

leucine aminopeptidase (EC.3.4.11.1.) is probably also present as it was detected both here and by Lüttge (1964a) although in both cases the activity is very low. The strong inhibition of the activity of crude secretion by EDTA supports the suggestion that these metallo-enzymes form an important component of the peptide hydrolase activity of the secretion. Some dipeptide hydrolases (EC.3.4.13.) are also metallo-enzymes and are inhibited by EDTA while many peptide hydrolases have a requirement for free metal ions and thus are inhibited by EDTA. Therefore the 68% inhibition caused by 20 mM-EDTA may be a summation of these two effects. Carboxypeptidase B (EC.3.4.12.3) does not appear to be present.

Acetylation with acetic anhydride causes over 40% inhibition of activity in the crude secretion suggesting that one of the major proteases present has an amide group involved in the active site. Pepstatin, the powerful pentapeptide inhibitor of pepsin and cathepsin D (Morishima et al, 1970), causes an increase in the activity of Dionaea secretion under conditions where it inhibits pepsin by 85%, suggesting that the catalytic action of Dionaea protease is not a peptic activity.

Thus it appears that the proteolytic package secreted by Dionaea does not contain either a serine protease (EC.3.4.21.) or a pepsin-like proteinase. It thus differs from the secretion of Nepenthes where the major proteinase activity in several species has been shown to be extremely like pepsin. In this genus the active site has been probed in both crude (Nakayama and Amagase, 1968; Amagase et

al, 1969) and partially purified secretion (Tökés et al, 1974) by analysing digest fragments to show the sites of specificity and treating with active-site directed inhibitors. Alkylation using p-chloromercuribenzoate (Nakayama and Amagase, 1968) or iodoacetic acid (Tökés et al, 1974) failed to inhibit nepenthesin as did acetylation with acetic anhydride or treatment with diisopropyl fluorophosphate or EDTA. Pepstatin, however, inhibited nepenthesin by 80% (Tökés et al, 1974).

The peptide digest pattern obtained using an extract from Drosera peltata leaves is, however, not at all similar to that from either pepsin or nepenthesin (Amagase, 1972). The analysis was conducted with crude secretion and hence cannot be attributed to a single proteolytic activity, but neither p-chloromercuribenzoate nor diisopropyl fluorophosphate caused any inhibition. Unfortunately pepstatin, metal-chelating agents and acetylation agents were not exhibited and thus it remains to be determined whether or not this proteolytic activity resembles that of Dionaea.

The secretion of Nepenthes and some Drosera species appears to possess more than one proteolytic activity, as in Dionaea. Zymograms prepared with crude secretion from mature pitchers of several species of Nepenthes show four bands of activity (Amagase, 1972) while the Sephadex G-25 elution profile of secretion from N. macferlanei shows two peaks of proteolytic activity (Tökés et al, 1974), the major one having been used for the active-site directed analysis discussed above. Lüttge (1964a) found that the activity of Nepenthes secretion differed when casein and

ovalbumin were used as substrate. He obtained a pH-activity curve against casein with peaks at pH2.0 - 2.5 and pH6.0 - 6.5 but only at pH2.0 - 2.5 with ovalbumin. He found leucine aminopeptidase and several di- and tripeptidase activities in the secretion of open pitchers but was undecided as to whether these were native to Nepenthes or due to microbial contamination.

Chandler and Anderson (1976b) have shown several pH optima for each of the proteolytic extracts from the leaves of three Australian species of Drosera. Amagase (1972) found that the leaf extract of D. peltata had a smooth pH-activity profile and only one band of activity on a zymogram. The use of extracts is, however, of little value when considering the activity of the secretion of these plants. The secretion of D. rotundifolia shows an irregular pH profile indicating the presence of more than one protease (Clancy and Coffey, 1977) but Whittaker (1949) found only one pH optimum for the hydrolysis of haemoglobin by secretion from both field-grown D. longifolia and aseptic cultures of D. filiformis. In all these cases, the major optimum pH peak is at pH2.2 - 3.0, considerably more acid than for Dionaea. Hence it seems likely that the activity of Drosera secretion is rather different from that of Dionaea but digest peptide sequencing studies are needed to examine this.

Although it has been demonstrated that proteolytic activity is released from the glands of Drosera (Palcewska, 1966), Drosophyllum (Heslop-Harrison, 1975), Pinguicula (Heslop-Harrison and Knox, 1971) and Utricularia (Vintéjoux, 1974a) and is present in the pitchers of Cephalotus (Dakin,

1919) and Sarracenia (Hepburn et al, 1927), there has been no recent work done on any of these genera to establish the nature of the proteolytic activity present.

2) Phosphatase Activity

Acid phosphatase activity was assayed here using p-Nitrophenyl phosphate but Scala et al (1969) showed several phosphatases to be present in the secretion. Whether esterase activity is physiologically important in the degradation of captured prey is questionable, particularly in view of the lack of any apparent sugar phosphatase activity in the secretion. Although the pH-activity profile (Fig. 6.1b) only shows one peak, the secretion separated by PAGE shows three activities (Fig. 6.2e). One of these (A) may be a polymeric form while band C could be due to partial proteolysis of band B. In Drosera rotundifolia, however, two pH optima are found at pH2.5 and 4.0 indicating that at least two acid phosphatases are present (Clancy and Coffey, 1977). Relative to p-Nitrophenyl phosphate, this secretion was 25 - 30% as active against the nucleoside 5'-di- and triphosphates at pH2.5 and 30 - 45% as active at pH4.0. The activity against several sugar phosphates and glycerol-1-phosphate was very low. Thus, with Dionaea, as with Drosera, much of the acid phosphatase activity may actually be due to the anhydrases, nucleoside di- and tri-phosphatases, and more than one acid phosphatase may be present.

The presence of these enzymes in other genera of carnivorous plant has not been investigated, apart from

the intra-glandular localizations discussed in Chapter 4.

3) Chitinase Activity

Chitinase, of high specific activity, is present in crude secretion. This enzyme has been found in the fluid of Nepenthes pitchers (Amagase et al, 1972) using both crushed and whole ants (Tetramorium caespitum) and colloidal chitin as substrates. Although these workers showed by thin-layer chromatography that the major product was N-acetyl-D-glucosamine, they did not quantify the results. They also reported chitinolytic activity in an extract from Drosera pedata leaves and Chandler and Anderson (1976b) found chitinase activity in leaf-extracts of D. whittakeri.

Hence this activity has been found in all the carnivorous plants so far examined. Chitin is extremely resistant to degradation and the activities of all these enzymes are low. A comparison of the chitinase from Dionaea secretion with that of Streptomyces griseus shows that Dionaea secretion has about one third the specific activity of the bacterial enzyme.

Chandler and Anderson (1976b) could not detect chitinase in leaf extracts from Drosera binata grown in axenic culture and from this conclude that the enzyme may be microbial in origin. With Nepenthes, however, Amagase et al (1972) used fluid from unopened pitchers, which is sterile, and so this activity is likely to be due to the proteins secreted by the plant. As no precautions to prevent microbial growth were taken with the material collected from Dionaea, the possibility that the chitinase

is due to contamination cannot be ruled out. When secretion is plated onto agar medium and incubated for several days, very few colonies of microbes develop indicating that the microbial concentration in secretion is very low. Indeed it is not impossible that the chitinolytic activity could be due to a lysozyme, secreted to inhibit bacterial growth. Scala et al (1969) tested the secretion for lysozyme using Micrococcus lysodeikticus but do not give their results. As no analysis of lysozyme activity was made in the present study, this proposal remains to be examined. Harder (1967), however, found that the secretion of several genera of carnivorous plants appeared not to contain any antiseptic agent.

It thus remains necessary to demonstrate that these enzymes are located intracellularly before they can be confirmed as of plant origin and not due to microbial contamination.

4) Peroxidase

Peroxidase was assayed as this enzyme can readily be localized to sub-cellular resolution using the 3,3'-diamino-benzidine reaction (Behnke, 1969). That peroxidase activity is present in the secretion is shown in Fig. 6.4. Due to various technical problems, it was not successfully detected in the gland cells. Hall and Sexton (1972) found peroxidase in small vacuoles and the cell wall of root cells but the distribution within the tissue is not the same as acid β-glycerophosphatase. Peroxidase also occurs in

abscission zones (Webster et al, 1976) where it is found in the cell wall together with acid phosphatase (Gilliland et al, 1976). In this tissue it is found, at early stages of abscission, in intracellular membrane-bound vacuoles (Webster et al, 1976) which are presumably involved in the secretion of these hydrolases. The role of peroxidase in abscission is not clear but it may be involved in ethylene production. Similarly the role of this enzyme in Dionaea activity is not obvious.

5) Conclusions

The package of enzymes contained within Dionaea secretion is very comparable to that found within various plant lysosomes (Matile, 1974) and especially the protein bodies of some seeds (Ory and Henningsen, 1969). This is not, perhaps, surprising as lysosomes are apparently a specially designed hydrolytic apparatus for, among other things, protecting cells against unwanted foreign matter and assimilating nutrients by endophagosis. There is good evidence that many specialized bodies such as vacuoles, aleurone grains and spherosomes are forms of lysosome while the hydrolase activity of the cell wall has led to the suggestion that this too is biochemically part of the lysosomal compartment (Matile, 1974).

It seems reasonable, therefore, to consider that the secretion may be lysosomal in origin. There are, however, certain differences. Various peptide hydrolases, glycosidases, esterases and anhydrases have been found in

lysosomes isolated by cell fractionation (Matile, 1974) but peroxidase does not appear to be present in any of these preparations. Furthermore, N-acetyl β-D-glucosaminidase (EC.3.2.1.30) is absent from Dionaea secretion, although this enzyme is considered to be a marker for lysosomes (Sogawa and Takahashi, 1977). Processing of the lysosomal package occurs in the development of both aleurone grains and spherosomes, however, therefore slight modifications to the hydrolytic package need not exclude the possibility that the secretion in Dionaea has evolved from this cellular system.

Type of Activity*			Substrate	Present	Absent	Source**
Peroxidase		EC1.11.1.7 Peroxidase	Hydrogen peroxide/guaiacol	+		2
Esterase		EC.3.1.3.2 Acid phosphatase	p-Nitrophenyl orthophosphate	+		2,3
		EC.3.1.3.19 Glycerol-2-phosphatase	Glycerol 2-phosphate		+	3
		EC.3.1.3.21 Glycerol-1-phosphatase	Glycerol 1-phosphate		+	3
		EC.3.1.3.23 Sugar phosphatase	Fructose 6-phosphate		+	3
			Fructose 1,6-bisphosphate		+	3
			Glucose 6-phosphate		+	3
		EC.3.1.4.5 Deoxyribonuclease	Deoxyribonucleic acid	+		3
Glycosidase		EC.3.2.1.1/2 Amylase	Dye-starch complex	+		3
		EC.3.2.1.14 Chitinase	Chitin	+		2
		EC.3.2.1.30 N-Acetyl-β-D-glucosaminidase	p-nitrophenyl-N-Acetyl-β-D-glucosaminidine		+	2
Peptide Hydrolase	Peptidase	EC.3.4.11.1 Leucine aminopeptidase	L-Leu-β-naphthylamide	+		1,2
		EC.3.4.12.2 Carboxypeptidase A	N-CBZ-L-ala-p-NPE	+		2
		EC.3.4.12.3 Carboxypeptidase B	N-Benzoylgly-L-arg		+	2
		EC.3.4.13.1 Glycyl-glycine hydrolase	Gly-gly	+		1
		EC.3.4.13.2 Glycyl-L-leucine hydrolase	Gly-L-leu	+		1
		EC.3.4.13.9 Proline dipeptidase	Aminoacyl-L-pro	+?		1
	Proteinase	EC.3.4.-.- General	Casein	+		1,2
			BSA	+		2
			Dye-fibrin complex	+(3)	+(2)	2,3
		EC.3.4.21.1 Chymotrypsin	N-CBZ-phe-p-NPE	+?		2
			ATEE		+	3
		EC.3.4.21.4 Trypsin	TAME		+	2,3
		EC.3.4.22.2 Papain	BAME		+	2
Anhydrase		EC.3.6.1.6. Nucleosidediphosphatase	Adenosine 5'-diphosphate		+	3
			Thymidine 5'-diphosphate		+	3
		EC.3.6.1.15 Nucleosidetriphosphatase	ATP	+		3
			Thymidine 5'-triphosphate	+		3

*The type of activity is defined as in Enzyme Nomenclature (1973), Elsevier Scientific Publishing Co., Amsterdam. pp.443
**1 = Lüttge (1964): 2 = Robins, present work, unpublished: 3 = Scala et al (1969)

Table 6.2: Enzymatic Activity of the Secretion from Dionaea Glands in the Presence of Various Inhibitors of Peptide Hydrolases

Inhibitor Present	Enzymatic Activity (% of Control)		
	Dionaea Secretion*	Pepsin*	Papain*
0.01 mM-pepstatin + 2.5%(v/v) ethanol	86.5	22.3	91.8
2.5%(v/v) ethanol	51.4	107.4	109.8
Pepstatin: corrected for ethanol	135.1	14.9	82.0
20 mM-EDTA	32.4	192.6	1339.3
10 mM-PMSF + 4%(v/v) propan-2-ol	64.9	70.3	83.6
4%(v/v) propan-2-ol	70.3	95.0	91.8
PMSF: corrected for propan-2-ol	94.6	75.3	91.8
0.5 mM-iodoacetamide	78.4	101.7	75.4
20 mM-acetic anhydride	56.8	114.1	267.2

*For the amounts used see 2:V:2.

Table 6.3: Enzymatic Activity of the Secretion from Dionaea Glands against Various Substrates Specific to Different Types of Peptide Hydrolase Activity

Type of Activity	Material	Conditions of Assay			Rate of Reaction ($\Delta E\ min^{-1}$ (ml solution^{-1}))
		Substrate	Additives	pH	
Leucine amino-peptidase EC.3.4.11.1	Secretion	0.4 mM-L-leu-β-naphthylamide		5.2	0.02
Carboxypeptidase A EC3.4.12.2	Secretion	0.3 mM-N-CBZ-L-ala-p-NPE		5.2	0.22
Carboxypeptidase B EC.3.4.12.3	Secretion	1.5 mM-N-Benzoylgly-L-arg		5.2 8.1	0.0 0.0
Chymotrypsin EC.3.4.21.1	Secretion	0.3 mM-N-CBZ-L-phe-p-NPE	77 mM-Ca^{++}	5.2	0.03
Trypsin EC.3.4.21.4	Secretion	1.0 mM-TAME	95 mM-Ca^{++} 95 mM-Ca^{++}	5.2 8.1	0.0 0.0
Papain EC.3.4.22.2	Papain (BDH) (1.0 mg ml^{-1})	50 μM-BAME	75 mM-Ca^{++} 75 mM-Ca^{++}:2.5 mM-EDTA 93 mM-Ca^{++} 93 mM-Ca^{++}:2.5 mM-EDTA 88 mM-Ca^{++}:25 μM-Iodoacetamide	5.2 5.2 6.75 6.75 6.75 6.75	0.0 0.0 3.5 3.7 5.4 0.1
	Secretion	50 μM-BAME	85 mM-Ca^{++} 82 mM-Ca^{++}:5.0 mM-EDTA 90 mM-Ca^{++}: 88 mM-Ca^{++}:5.0 mM-EDTA 88 mM-Ca^{++}:1.3 μM-Iodoacetamide	6.75 6.75 5.2 5.2 5.2	0.0 0.01 0.0 0.0 0.0

Figure 6.1 The pH-activity profile obtained using crude secretion when assayed for:

a) Acid protease activity;

b) Acid phosphatase activity.

● 30°C

■ 25°C

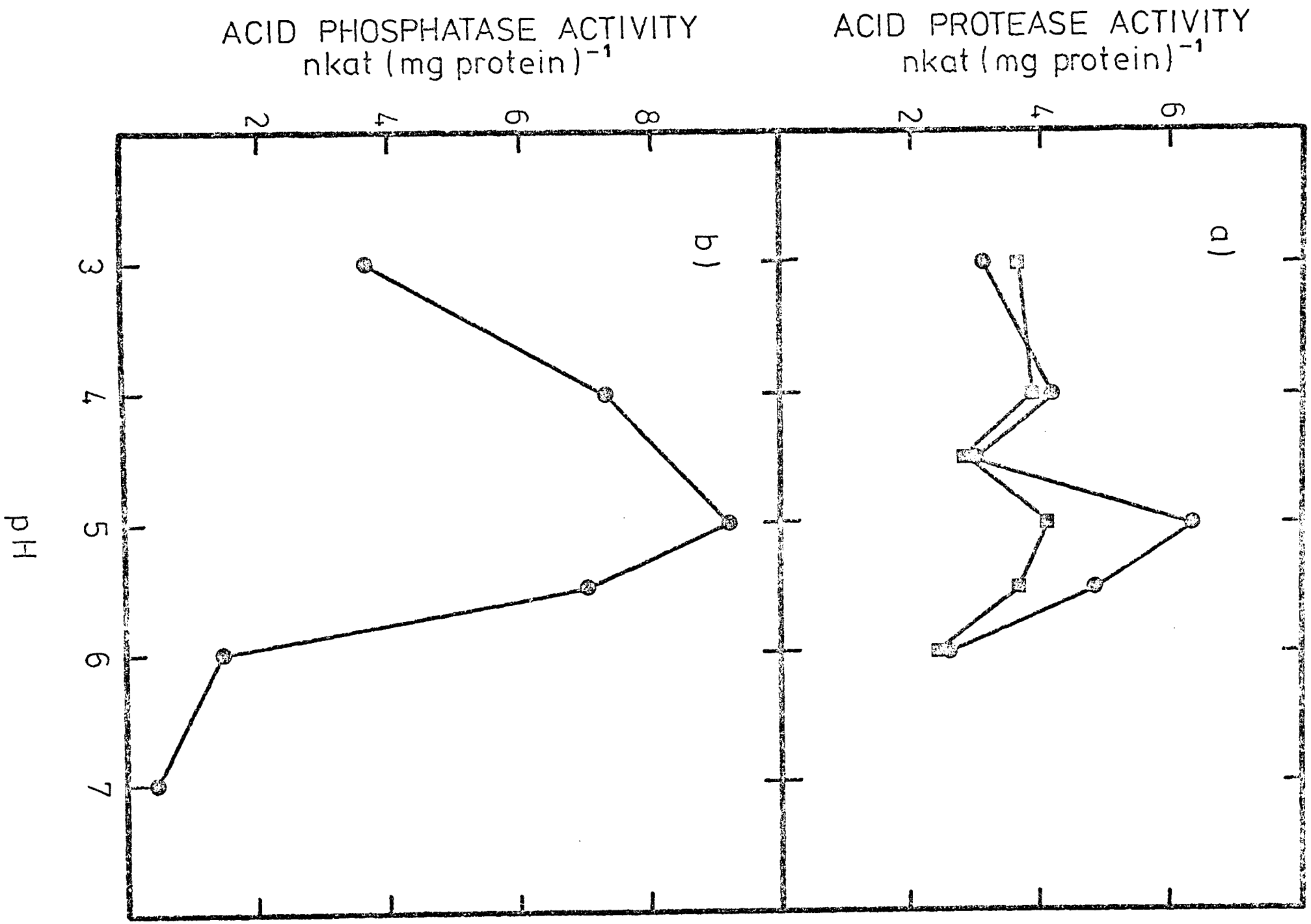

ACID PHOSPHATASE ACTIVITY
nkat (mg protein)$^{-1}$
ACID PROTEASE ACTIVITY
nkat (mg protein)$^{-1}$
2
4
6
8
2
4
6
b)
a)
3
4
5
6
7
pH

Figure 6.2 Electrophoretic patterns obtained using crude secretion under various conditions of PAGE as described in the Methods (2:III:2).

a) Total protein - with SDS - whole gel in Coomassie blue;

b) Acid protease - without SDS - whole gel zymogram;

c) Acid protease - with SDS - whole gel zymogram;

d) Acid phosphatase - with SDS - whole gel zymogram;

e) Acid phosphatase - with SDS - gel sliced and sections incubated.

Running conditions:

Gel	Load of Secretion (µl)	Time (min)	Length of gel (mm)	Buffers
a	50	190	103	all: 25 mM-sodium acetate containing 0.1%(w/v) SDS
b	40	40	60	cathode chamber: 50 mM-sodium acetate (pH5.0). gel and anode: 50 mM-sodium acetate (pH5.0) containing 0.75%(w/v) casein.
c	50	190	101	as 'a' but including 0.75% (w/v) casein.
d	50	190	98	as 'a'.
e	100	190	98	as 'a'.

In all cases electrophoresis was conducted at 4 mA $tube^{-1}$ in the cold.

+ −

a)

A B C D

b)

A BC D

c)

A B C D

d)

e)

A B C

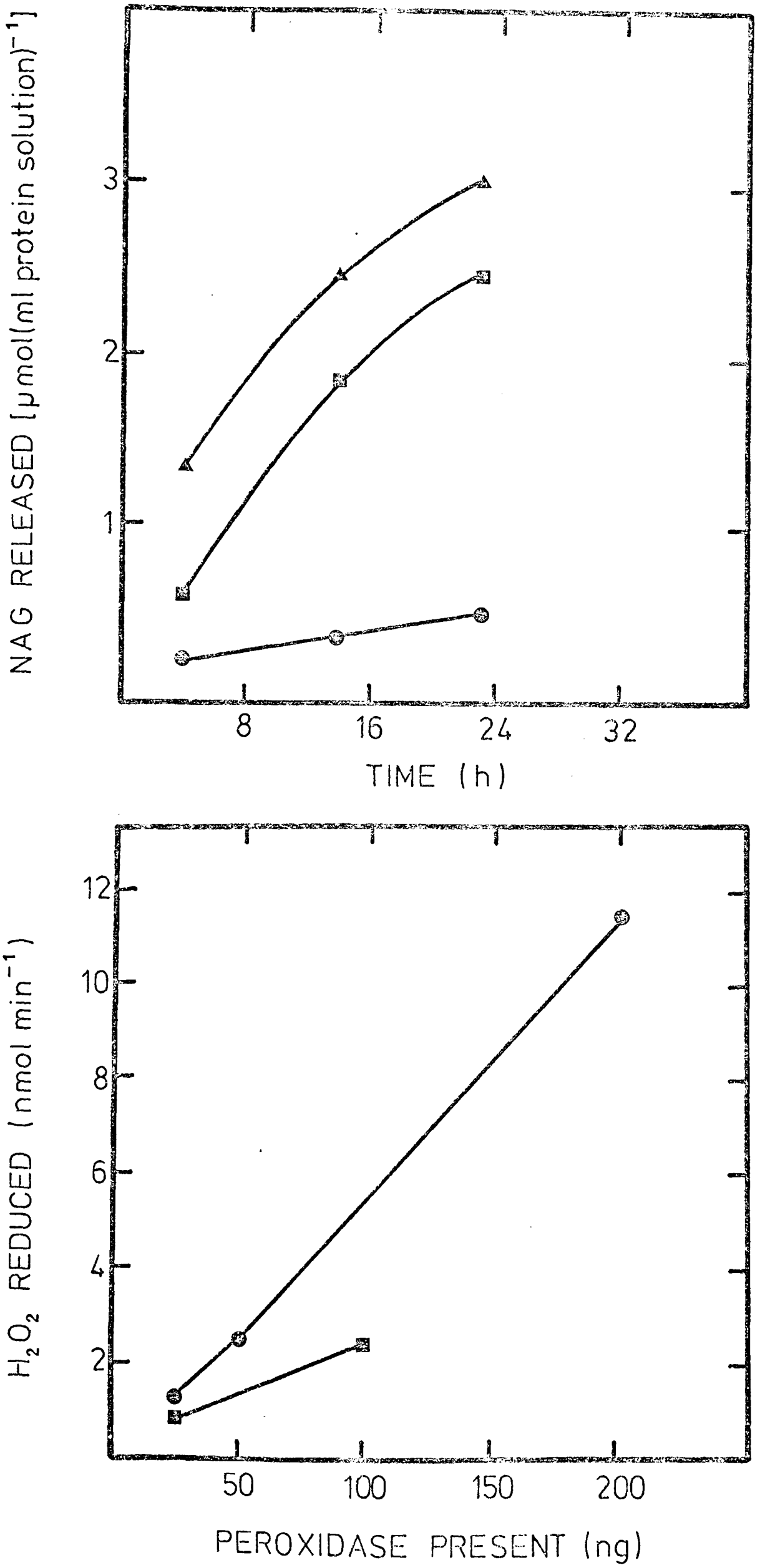
A B C

Figure 6.3 Chitinase activity: plotted as the rate of release of NAG from colloidal chitin (120 mg ml^{-1}).

- ● 100 µl crude secretion (2.5 mg protein ml^{-1}) per ml incubation medium. pH5.0
- ■ 100 µl SCC chitinase (6.0 mg protein ml^{-1}) per ml incubation medium. pH6.0.
- ▲ 100 µl SCC chitinase (6.0 mg protein ml^{-1}) per ml incubation medium. pH5.0.

Figure 6.4 Peroxidase activity in crude secretion.

- ● pH5.0
- ■ pH7.0


NAG RELEASED [μmol(ml protein solution)⁻¹]
1
2
3
8
16
24
32
TIME (h)
H_2O_2 REDUCED (nmol min⁻¹)
2
4
6
8
10
12
50
100
150
200
PEROXIDASE PRESENT (ng)

CHAPTER 7

THE ABSORPTION OF NUTRIENTS

I INTRODUCTION

II RESULTS

1) Uptake of Vital Dyes

2) Uptake and Assimilation of Radiolabel

3) Uptake of Chloride Ions

4) Ultrastructural Aspects of Absorption

III DISCUSSION

1) The Absorption and Distribution of Tracer Compounds

2) Ultrastructural Aspects of Absorption

IV APPENDIX : Theoretical Considerations of the Estimation of Plasmodesmatal Frequencies

I INTRODUCTION

Darwin (1875) observed that the glands of many genera of carnivorous plants, including Dionaea, are capable of absorbing nutrients through the leaves. Lüttge (1963) showed Dionaea leaves to absorb [^{14}C]-glutamine, $^{35}SO_4^{--}$ and $^{45}Ca^{++}$ and that within 24 h the radiolabel was distributed throughout the leaf. Subsequently, he showed the absorption of several compounds to be accompanied by elevated respiration (Lüttge, 1964b) and, by autoradiography using $^{35}SO_4^{--}$, that absorption by both Nepenthes and Dionaea took place exclusively via the glands, none of the absorbed substances passing through the normal epidermal cells (Lüttge, 1965).

Arisz (1953) showed that in Drosera the absorption of various substances by the stalked glands was energy-dependent and Helder (1967) summarizes the evidence from this and other work which shows that transport by these glands is active. It is also rapid. When L-[^{3}H]-asparagine is applied to the gland head radiolabel may be detected by autoradiography after only 2 min in the head of the gland and within the entire tentacle after 60 min (Gilchrist, 1974; Juniper et al, 1977). The subcellular pathway for the absorption of calcium by these glands, when fed with milk, was followed using X-ray microscope analysis (Gilchrist, 1974; Juniper and Gilchrist, 1976), calcium also being detected in the head of the gland after only 2 min.

The only other investigation of uptake by a carnivorous plant at the subcellular level is that of Heslop-Harrison

(1976) who showed that colloidal lanthanum II nitrate is absorbed by the sessile glands of Pinguicula, while previously Heslop-Harrison and Knox (1971) had shown Pinguicula to absorb ^{14}C-label from [U-^{14}C]-protein within 4 h of application.

The absorption of nitrogenous matter by all three genera of the Sarraceniaceae (Sarracenia, Darlingtonia and Heliamphora) was demonstrated by Hepburn et al (1920) while Plummer and Kethley (1964) showed Sarracenia to be capable of absorbing both amino acids and peptides. They further found that the rates of absorption varied for different L-amino acids but did not demonstrate whether or not this process was energy-dependent.

It has been shown in several carnivorous genera that the foliar absorption of nutrient is advantageous to the plant. Several early workers (Lloyd, 1942: p.162-165) demonstrated that plants of Drosera which were able to capture prey grew more vigorously and set a greater amount of seed than those from which prey was excluded. Harder and Zemlin (1968) found Pinguicula grown in axenic culture to grow and flower more vigorously when fed pollen and Harder (1970) found that the dry weight of Utricularia increased more rapidly in plants fed Daphnia than in the unfed controls. Fabian-Galan and Salageanu (1968) were able to demonstrate that when ^{14}C-labelled Daphnia were fed to Drosera and Aldrovanda radiolabel could be detected in the leaves of these plants, and this has been confirmed with ^{35}S-labelled Drosophila fed to Drosera (Chandler and Anderson, 1976c).

Clearly, then, many and probably all the genera of

carnivorous plants are capable of absorbing material through their leaf-surface glands, and it appears that both salts and organic molecules are absorbed, probably by energy-dependent processes.

Absorption by Dionaea was not studied extensively here and is restricted to the pathway of chloride absorption, a demonstration that radiolabel is taken into gland cells and incorporated into the plant tissue and a consideration of the role of those ultrastructural features which may be involved in the movement of assimilated matter into these plants.

II RESULTS

1) Uptake of Vital Dyes

The vital stain methylene blue (0.01%(w/v)) is rapidly taken into glands when applied to the adaxial surface of a stimulated trap. Within 1 min slight penetration into the gland is visible; by 2 min the cytoplasm is stained while the vacuole remains clear; by 5 min the whole gland head is heavily stained, although the vacuole is much less intensely stained than the cytoplasm. No penetration of this dye occurs through the non-glandular cells of the epidermis.

2) Uptake and Assimilation of Radiolabel

The data presented in Chapter 4 (4:II:7) show that the glands are capable of absorbing radiolabel and assimilating it into proteins. Radiolabel occurs in proteins within 24 h but the time-course for the absorption of L-leucine was not examined.

3) Uptake of Chloride Ions

When glands are fed 2 or 4%(w/v) bactopeptone solution containing 100 mM-sodium chloride, it is found that this chloride is absorbed by the glands of the leaf surface. Fig. 7.1 shows that within 2 h silver I chloride precipitates are observed throughout the gland head and in the basal cells. Intense deposition occurs associated with the core material

of the labyrinthine wall (Fig. 7.2) and the vacuole is also intensely stained. Small deposits occur throughout the cytoplasm, notably associated with mitochondria (Fig. 7.2). Within the stalk cells, deposits are absent from the lipid droplets (Fig. 7.1).

After three days feeding with chloride, the core material of the labyrinthine walls no longer shows any deposits but instead deposits occur along the plasmalemma and middle lamella of the wall (Fig. 7.3 : 7.4). The vacuoles stain intensely with osmium and still contain numerous silver I chloride deposits. They have become highly amoeboid, apparently engulfing large regions of cytoplasm including mitochondria and endoplasmic reticulum (Fig. 7.5). The cytoplasm of these cells is very poorly defined and contains few deposits. Within the stalk cell, heavy deposits of silver I chloride are found along the cell walls (Fig. 7.6) and particularly juxtaposed to the endocuticle, which contains very few grains (Fig. 7.7). The plasmodesmata between the stalk and basal cells contain heavy deposits while the wall regions are largely free of these (Fig. 7.8) and there are heavy deposits along the edges of the wall (Fig. 7.9). The pit fields show intense deposition (Fig. 7.9). Control tissue fixed without silver I nitrate present shows no such deposits.

4) Ultrastructural Aspects of Absorption

1. The labyrinthine cell wall. As already mentioned in Chapter 3, the labyrinthine cell wall shows marked polarity

across the gland with a greater ingrowth density and number of ingrowths in the inner gland cells (Fig. 3.37). Fig. 7.10a shows the mean amplification factors at the various gland boundaries for measurements taken from all stages of the secretory cycle. There is no significant difference (χ^2-test) between these mean values and those for days 0, 2, 4, and 6 individually. There is however a significant difference (see legend to Fig. 7.10) between the amplification factor of each boundary and that to either side, except for the comparison of the two sides of the wall between the outer and inner secretory cells.

Fig. 7.10b shows the total area of each boundary within the gland, as measured in epoxy-embedded tissue in the light microscope (see 2:II:11) and the total area of transfer surface available at each of these boundaries when the amplification factor is taken into account.

2. Plasmodesmata. There are certain serious, and as yet unresolved, problems concerning the quantification of plasmodesmatal frequencies. These are considered in an Appendix to this chapter. Fig. 7.11 shows the frequency observed in the different cell/cell boundaries of the gland head when it is assumed that 0.76 of the external radius of the plasmodesma must be visible for it to be recorded. The validity of this assumption and the effect it has on the actual number of plasmodesmata are considered in the Appendix. Alterations of the frequency function have only a small effect on the relative values of these frequencies but considerable effect on the absolute values.

The number of plasmodesmata per unit area shows a strong polarity across the gland, as shown in Fig. 7.11a. As there are none in the exterior wall, the ratio of this increase cannot be calculated. The frequency in the outer/outer secretory cell wall is 0.13 μm^{-2} and using this value a ratio to the inner secretory/stalk cell wall of 29:1 is obtained. The total number of plasmodesmata in the inner/outer secretory cell wall is x 2.3 the number in the inner secretory/stalk cell wall but only x 1.2 the number between the stalk and basal cells. The frequency out of the basal cell has not been determined.

The area of wall occupied by plasmodesmata at the inner secretory/stalk cell boundary is 7.95 μm^2 and at the inner/outer secretory cell boundary is 10.93 μm^2. The diameters of the plasmodesmata in the different boundaries vary considerably and are given in Table 3.3.

III DISCUSSION

The same glands that secrete the digestive fluid are simultaneously involved in absorbing the small-molecular products of the hydrolase activity. The pathway of this absorption has yet to be established. Conventional high-resolution autoradiographic preparation does not satisfactorily localize small molecules as these are lost from the tissue during preparation, while cryostatic methods are fraught with equally great problems. Nevertheless, cryostatics is probably the only method by which the fine-structural details of the pathway of absorption of digestive products might be examined.

1) The Absorption and Distribution of Tracer Compounds

The use of tracers such as calcium (Gilchrist, 1974; Juniper and Gilchrist, 1976), colloidal lanthanum II nitrate (Heslop-Harrison, 1976; Thomson et al, 1973), hexacyanoferrate III (Pizzolato et al, 1976), bromide (Van Steveninck et al, 1976c) or chloride (Van Steveninck and Chenoweth, 1972; Van Steveninck et al, 1976a, b) will only show the pathway of movement for that ion or compound and should not be interpreted in more general terms. Furthermore, any interpretation requires considerable care. For example, colloidal lanthanum II nitrate was initially developed as a tracer to delineate the apoplastic space (Thomson et al, 1973) as it is not apparently carried across the plasmalemma, yet subsequently it has been interpreted as showing that the route of

absorption is via the apoplast (Heslop-Harrison, 1976).

The heavy concentrations of silver I chloride deposits which occur within the walls and vacuoles of Dionaea gland secretory cells within 2 h of adding chloride ions to the stimulating solution show that this ion is rapidly taken into the gland head. It is apparently concentrated into the vacuole, although some of the deposits seen are probably due to endogenous chloride ions. Initially, chloride ions seem to be held by the core material of the labyrinthine walls but after three days treatment with 100 mM-sodium chloride (by which time the ionic balance of the cells is presumably severely disrupted) these are no longer present and deposits are only found in the walls along the plasmalemma or the middle lamella. The cause of this alteration is not clear but could be due to different aqueous and ionic balances in the leaves of plants saturated with chloride and those to which the ion has only just been introduced at high concentration.

Heslop-Harrison (1976) found that lanthanum II nitrate does not penetrate the endocuticle in Pinguicula and similarly in Dionaea chloride ions seem to be largely excluded from this part of the wall (Fig. 7.7), suggesting that it presents a block to apoplastic flow. Although the cell walls between the secretory cells appear permeable to chloride ions, those between the stalk cell and the basal cell do not. A concentration of deposits may be seen along the stalk cell side of this boundary (Fig. 7.6) and only passing through the wall where there are pit-fields containing plasmodesmata (Figs. 7.8 : 7.9). Some ordinary

sections (see, for example, Figs. 3.6 : 3.14) show a dark-staining deposit, continuous with the endocuticle, within this wall. This is very probably a suberized layer, as found in the wall between the mesophyll and bundle-sheath cells of wheat and oat (O'Brien and Carr, 1970), and will restrict metabolic exchange across this wall to the symplast. The extremely intense deposits at the stalk cell side of the pit fields suggest that the ions are moving in a direction away from the secretory cells. Van Steveninck and Chenoweth (1972) and Van Steveninck *et al* (1976a) find similar images in barley seedling parenchyma cells treated with 100 mM-sodium chloride (external concentration) with concentrations of silver I chloride deposits along the walls, in the plasmodesmata and in the vacuoles, where they are particularly associated with the tonoplast. Endogenous chloride ions in the mesophyll cells of the mangrove *Aegiceras corniculatum* (Van Steveninck *et al*, 1976b) and the salt-gland cells of *Limonium vulgare* (Ziegler and Lüttge, 1967) show a similar pattern of distribution while in *Limonium* large deposits also occur around the points of discharge through the cuticle. In *Sueda maritima* chloride is primarily located in the vacuole (Harvey *et al*, 1976).

An association of chloride ions with the plasmalemma appears common and may represent ions bound to proteins within the matrix of the membrane. It is not entirely due to transport of these ions from one cell to another as a similar distribution occurs in *Nitella* cells (Van Steveninck *et al*, 1976c). As a redistribution of ions may occur during fixation (Komnick and Bierthier, 1969) the

subcellular localization might, however, be grossly distorted. Furthermore, if for example, the cell wall between the stalk and basal cells was freely permeable to chloride ions but impermeable to silver I ions then the latter, and the deposits of silver I chloride, would be confined to the plasmodesmata. Similarly, if the cationic silver I ions were initially bound to the negatively-charged phospholipids of the plasmalemma and then reacted with chloride ions as these leached out of the pre-loaded cells (no chloride ions being present in the fixative solutions) this would produce the pattern of heavy deposits along the plasmalemma observed in this gland and the other systems cited above. Hence in those systems where chloride is not endogenous it becomes difficult to distinguish real distributions from preparative artefacts. Neither controls nor the use of X-ray microprobe analysis can help with this problem.

The only unequivocal conclusion from these experiments, therefore, is that chloride ions rapidly enter the gland and are distributed throughout all the gland cells within 2 h. In addition, it appears that chloride ions cause the large osmiophilic vacuole to adopt a much more amoeboid shape than usual and possibly to engulf small regions of cytoplasm. Furthermore, the images suggest that in the cell walls between the auxiliary cells these ions migrate through the plasmodesmata and not through the apoplast.

The time-course of absorption of methylene blue shows that, in the stimulated state, the glands will very rapidly absorb substances and that these may quickly enter the cells.

The incorporation of radiolabelled leucine into secretory proteins shows that the glands will absorb amino acids, while the data presented in Table 4.5 show that most of the radiolabel within the leaves is in the TCA-precipitable fraction.

2) Ultrastructural Aspects of Absorption

The absence of pores from the cuticle suggests that in Dionaea glands digestive products are absorbed throughout the gland-head surface. The cuticle is thicker than in the stalked glands of Drosera (Ragetli et al, 1972) but very much thinner than the epidermal cuticle of Dionaea. In the sessile glands of Pinguicula, colloidal lanthanum II nitrate readily passes through the cuticle into the cellulosic and labyrinthine cell walls but will not penetrate the cuticle of the epidermal cells (Heslop-Harrison, 1976). A thin cuticle is a feature of many secretory glands but is apparently also important for absorption. It does not require discontinuities to be absorptive, as cuticular pores are not visible in Dionaea yet this gland is capable of absorption. The only indication that the cuticle may not be completely uniform is found in Fig. 7.4 where heavy deposits of silver I chloride are found at discrete points along the external cell wall, suggesting that differential absorption of the silver I ions may be occurring. The validity of such interpretations, however, has already been questioned (7:III:1) and, furthermore, such deposits are only rarely seen.

The endocuticle has already been mentioned as a consistent feature of plant glands (3:IV:2). Heslop-Harrison (1976) found with the sessile glands of Pinguicula that the apoplastic tracer lanthanum II nitrate was excluded from the endocuticle even though it is unable to pass through the intervening cells. Similarly, in the stalked glands of Drosera when calcium was used as a tracer (Gilchrist, 1974; Juniper and Gilchrist, 1976) it was excluded from the endocuticle although present throughout the rest of the apoplast and in the symplast. In Dionaea, this suberized wall is apparently impermeable either to chloride or to silver I ions (Fig. 7.7). In Dionaea then, as in other glands, all communication between the gland and the leaf tissue is very probably entirely through the symplast.

Absorbed substances, then, must at some point pass into the symplast within the gland. It has already been shown in Chapter 4 that some of this material is utilized within the secretory cells but the number of counts min^{-1} obtained was always much below the level of radiolabel applied to the traps, indicating that the majority of absorbed material was probably passing through the gland to be used elsewhere within the plant. As detailed in the Introduction to this chapter it has been found in other genera that plants fed with nitrogenous material through the leaves grow more vigorously than those which are not. It was found during the course of this study that plants which were able to capture prey grew more vigorously than those which were prevented from so doing.

The gland head is very asymmetrical, with an exterior

surface area x 10 that of the stalk/basal cell boundary (Fig. 7.10). Therefore, either the rate of absorption of nutrients will be restricted by the structural constriction at the stalk cells (Fig. 3.5), or the absorptive system of the gland must be designed so as to overcome this problem.

Two possible pathways of absorption present themselves:

(i) Material may adopt a primarily apoplastic flow through the two layers of secretory cells, only entering the symplast at the stalk cell surface.

(ii) Material may pass across the plasmalemma of the secretory cells and mostly move through the gland in the symplast.

The key features of each of these pathways are, respectively, the areas of plasmalemma available for absorption at the different cellular layers and the density of plasmodesmata within the different cell boundaries throughout the cell.

If only pathway (i) is operational, then to maximize the efficiency of uptake, the surface area at the stalk cell boundary must have an uptake capacity equivalent to the exterior surface of the gland. This could be achieved either by having a highly labyrinthine wall to maintain a constant absorptive area or by greatly increasing the density of transport systems within the membrane.

That the former possibility is not the case is shown by Fig. 7.10a. The surface of the cell wall in the stalk cells which faces the inner secretory cells is noticeably devoid of labyrinthine protruberances and has a total area

of only 8 - 10% of the exterior surface of the gland.

The concentration of transport systems is unknown. The localization of lead II phosphate deposits along the plasmalemma from incubation with β-glycerophosphate or ATP (see 4:II:1), however, may give some indication of this (Figier, 1968a). With neither substrate was a heavier deposit obvious at one cell surface of the gland than elsewhere. This suggests that no particular plasmalemma has an exceptionally high concentration of phosphatase activity. The results from the chloride tracking suggest that this ion is not present in large concentrations within the inner secretory/stalk cell wall (Fig. 7.5) but as always such evidence must be treated with caution.

If only pathway (ii) were operational then it might be expected that the greatest absorptive surface would be at the exterior of the gland and that there would be a system of plasmodesmata capable of maintaining a constant flux across the rest of the intercellular boundaries. Although the frequency of plasmodesmata per μm^2 increases 30-fold from the outermost to the inner side of the gland, the total number between the inner secretory cells and the stalk cells is only half that between the outer and inner secretory cells (see Fig. 7.11). In the former boundary, however, the plasmodesmata are of considerably greater diameter than those in the latter (Table 3.3) and thus the total area of wall occupied by plasmodesmata between the stalk and inner secretory cells is 7.95 μm^2 while that between the outer and inner secretory cells is 10.93 μm^2, only x 1.4 higher. An examination of the flux kinetics through plasmodesmata is

beyond the scope of the present discussion, but may be found in Gunning and Robards (1976). What is, however, highly relevant is that if the plasmodesma is treated as a cylindrical channel through which fluid flows in a Newtonian fashion then, all other parameters being invariant, the rate at which the fluid passes through the plasmodesma is dependent on the fourth power of the radius (r^4). Hence under these conditions, the ratio of the rate of fluid flux through a plasmodesma in the inner secretory/stalk cell wall to that in the outer secretory/inner secretory cell wall will be 3.27. It is highly improbable that other parameters such as the viscosity of the fluid and the pressure drop along the plasmodesma will remain constant and such a statement also ignores the effect of the desmotubule. Even a ratio of only half this, however, will adequately compensate for the smaller number of plasmodesmata in the inner secretory/stalk cell wall.

Thus there is apparently sufficient capacity in the plasmodesmatal system to cope with a flux through the gland which is entirely symplastic. Nevertheless, for this to be the complete picture is incompatible with the observed distribution of labyrinthine walls which, as mentioned earlier, is not as might be expected if this wall component were entirely involved in the secretory activity of the gland.

Hence it is proposed:

a) That absorbed substances may migrate across the secretory-cell layers in either the apoplast or the symplast.

Apoplastic movement will be entirely dependent on diffusion along concentration gradients and on the mass flow of fluid, whereas in the symplast migration will be subject to metabolic control.

b) That across the inner secretory cell/stalk cell and the stalk cell/basal cell boundaries the flow is entirely symplastic. To ensure that the flow rate is not disrupted by the limited number of plasmodesmata at the former of these boundaries, each channel is greater than those in the secretory cell layers.

c) That throughout the secretory-cell layers the flow is predominantly symplastic. The architecture and disposition of the plasmodesmata is such as to enable a flow across the gland which is not limited by a shortage of inter-cellular channels.

d) That the labyrinthine walls are important in ensuring that the absorbed material is rapidly assimilated into the symplast by offering a high absorptive area.

e) That the polarity of the labyrinthine wall is such that the area of plasmalemma within the gland is comparable to or greater than the area at the surface. Thus (assuming that the concentration of translocating systems within the membranes does not significantly alter) by actively transporting material into the inner cells of the gland, a concentration gradient within the apoplast can be established and maintained, which will increase the rate of diffusion of material into the gland. This will enable an inward flux of digestive products and an outward flux of secretory

proteins to occur simultaneously which will be independent of any mass flow.

The absorption of material is probably an active process as shown for Drosera by Arisz (1953) and suggested by the increased respiration during absorption found in Dionaea by Lüttge (1964b). It is apparently not by reversed pinocytosis or by phagocytosis as there is no ultrastructural evidence to substantiate a passage of vesicles from the cell boundary into the cytoplasm. Once within the cells, the absorbed metabolites may be concentrated into the small clear vacuoles which appear after stimulation and are not apparently involved in the storage of secretory proteins. These vacuoles become very much more common after stimulation and persist at an elevated level even towards the end of the cycle (Fig. 3.44). They are frequently seen fusing one to another and with small vesicles fusing to them (Fig. 3.19), although the origin of these vesicles is not clear. To confine the digestion products to such vacuoles might help to maintain some diffusion gradients into the cell and to prevent leakage. It would also make further intracellular hydrolysis of partially-degraded products possible. As these vacuoles cannot pass through plasmodesmata, the metabolites would need to be released back into the cytoplasm to migrate to the next cell. Much further work, however, is required to establish their role. All intracellular aspects of the absorption process are at present purely speculative and need to be examined by cryostatic methods.

As detailed in the Introduction (7:I), several

investigators have studied absorption in carnivorous plants (also see Lloyd (1942) for an examination of earlier work) but only two other studies have entailed a subcellular level of resolution.

Gilchrist (1974) and Juniper and Gilchrist (1976) have presented similar proposals for the transport of calcium through the stalked glands of Drosera. Here too a combined system of apoplastic and symplastic flow apparently occurs. Table 1 of Juniper et al (1977) summarizes the data of Gilchrist (1974). After 1 - 2 min feeding, calcium is found predominantly in the outer secretory cell layer but evenly distributed between the cytoplasm and the wall, whereas after 15 min it is mostly in the cytoplasm. After 60 min, however, the cytoplasms and cell walls of both secretory cell layers are loaded with calcium. This suggests that uptake into the symplast occurs immediately and not only as a result of a high concentration in the apoplast.

The polarity of the wall ingrowths in Drosera is similar to that of Dionaea which also suggests that uptake across the secretory layers may be similar. Beyond the endodermal cells, however, the structures of the two glands are quite different and the blebbing of the plasmalemma at the face of the endodermal cells facing the tracheid mass seen by Gilchrist and Juniper (1974) is never found in Dionaea.

Working with Pinguicula, Heslop-Harrison (1975; 1976) finds colloidal lanthanum II nitrate to be absorbed by the sessile glands and to pass through the apoplast and from this proposes a model of secretion and absorption based on mass flow through the gland. She considers, however, that

"it is inconceivable that the glands could permit a two-way traffic, so it must be supposed that, in each, the phase of secretion must give place quite rapidly to that of absorption" (Heslop-Harrison, 1976). Such a view may well be an oversimplification of the activity of these glands which appear not to be as complex as those in Dionaea and do not recover after one sequence of operation.

As she points out, such a mechanism is not applicable to Dionaea (Heslop-Harrison, 1976) where two-way traffic does occur and the glands remain active. Instead the glands of Dionaea appear to have developed a system which uses active transport throughout the absorptive surfaces of the gland to create a favourable concentration gradient in the apoplast. Once in the symplast the carrying capacity of the plasmodesmata is sufficient to maintain a steady flux of absorbed material out of the gland into the leaf-tissue.

It has already been noted in Chapter 3 (3:IV:2) that the distribution of labyrinthine walls found in the bifid and quadrifid glands of Utricularia by Fineran and Lee (1974a, b; 1975) is very different from that of Dionaea. In these much simpler glands it is the stalk cell (which, in their terminology, is the pedestal cell) which contains labyrinthine walls and not the cells forming the cap of the gland. Sydenham and Findlay (1975) have found that, during the resetting of the trapping-bladders of Utricularia, ions (mostly sodium, potassium and chloride) are rapidly and actively transported from the lumen of the trap into the external medium. On the basis of this they suggest a mechanism in which chloride ions are actively pumped from

the lumen into the cells of the bladder drawing with them water and counter-ions. Both ions and water then passively pass through the sub-cuticular space into the external medium. It is suggested in both these publications that the glands are involved in this water movement. This is very probably the case and the subcellular architecture of the glands, examined by Fineran and Lee (1974a, b; 1975), is completely compatible with the pathway of absorption in these glands being by the alternative (pathway (i)) to that suggested for Dionaea glands. The cap cells have thick cell walls, particularly near to the pedestal cell, and the matrix of these walls appears diffuse while the cuticle is very thin. Within the pedestal cell there are extensive labyrinthine walls arising almost entirely on the boundary facing the cap cells, although at times they may be traced right across the cell. In the pedestal/basal cell boundary are numerous plasmodesmata, while the numbers of these in the pedestal/cap cell boundary is apparently much lower. The wall surrounding the pedestal cell is heavily impregnated, as in so many glands, forming an impermeable endocuticle. Thus, as suggested by Fineran and Lee (1975), the subcellular structure of these glands is highly suited to a pathway of absorption in which absorbed substances flow through the apoplast of the cap cells, enter the symplast at the pedestal cell and remain in the symplast as they migrate into the bladder tissue.

IV APPENDIX

Theoretical Considerations of the Estimation of Plasmodesmatal Frequencies

The number of plasmodesmata per μm^2 of a cell/cell boundary (F) is an important factor when considering the relationship of a cell or group of cells to any neighbouring cells. To obtain this factor requires several measurements of the material prepared for the electron microscope. These are:

(i) The number of plasmodesmata per μm of length of wall;

(ii) The diameter of each of these plasmodesmata;

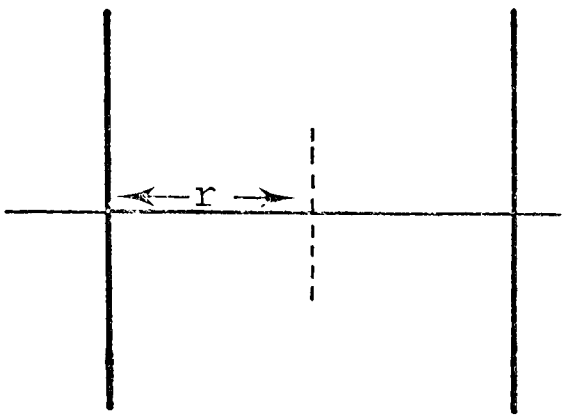
(iii) The thickness of the sections from which (i) and (ii) are obtained.

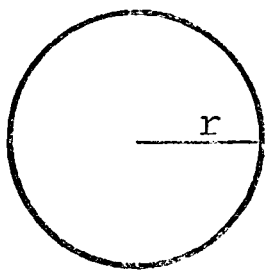
For the purpose of these considerations, plasmodesmata are taken as cylindrical structures of uniform maximal diameter 2r, where 2r is measured to the exterior of the tubule membrane. It is accepted that plasmodesmata are not of uniform cross-sectional diameter, neither one to another nor along their length, but the introduction of the relevant correction factors at this stage will only serve to complicate the basic analysis.

Normally plasmodesmata are seen in longitudinal section (LS) while transverse sections (TS) are much more rarely attained. TS have the advantage that no estimate of (iii) is required but the disadvantage that there is no way of ensuring that all the sections are truely TS and not at an angle to the axis of the plasmodesma. To what extent, then, are the observed values affected by tilting the plane of

section so as to cut the central axis of a plasmodesma at an angle θ away from the perpendicular?

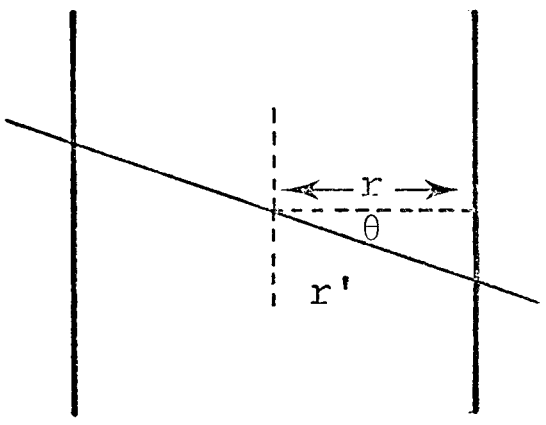
1) at 90°:

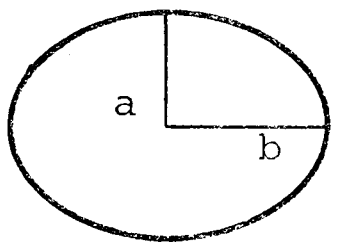




$\text{area} = \pi r^2$

2) at $90^{\circ} + \theta$:





$a = r + \delta r_1$

$b = r + \delta r_2$

The assumption is being made that this is still a circular cross-section and the area is calculated in terms of observed values a or b.

If r' represents half the measured diameter then

$$\cos\theta = r/r' \qquad 1.$$

i.e. $$r' = r/\cos\theta = r \sec\theta \qquad 2.$$

Thus:

$\theta(^{\circ})$	0	5	10	15	20	30	45
r'	r	1.004r	1.015r	1.035r	1.069r	1.155r	1.414r
Relative area	1.000	1.008	1.030	1.071	1.142	1.334	1.999
% error	0.0	0.8	3.0	7.1	14.2	33.4	99.9

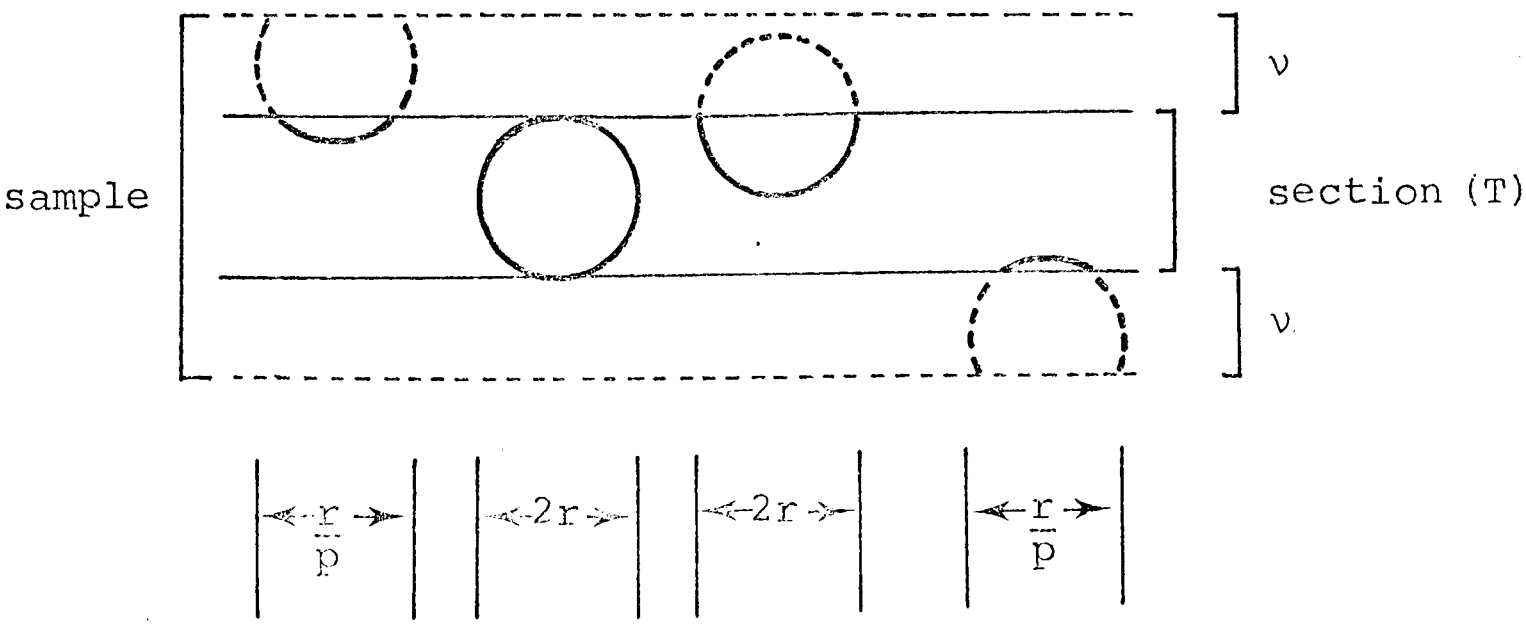
Hence as small a tilt in the cutting plane as 15 - 20^{o} will cause an error of approximately 10% in the calculation of the area of that plasmodesma. Furthermore, as the angle increases then the introduced error greatly escalates. Because this angle cannot readily be estimated, TS are not satisfactory for determining F.

When viewing plasmodesmata in LS the major problem is introduced by the relative thicknesses of the section and the plasmodesmatal channels. Silver/gold or silver sections have a thickness of 70 - 100 nm (Peachey, 1958) while plasmodesmata range from about 25 - 100 nm in diameter (Robards, 1976). Thus when all plasmodesmata visible in a section are counted, the sample size is actually greater than the section thickness by a factor which is itself dependent on the diameter of the plasmodesmata present. Hence if:

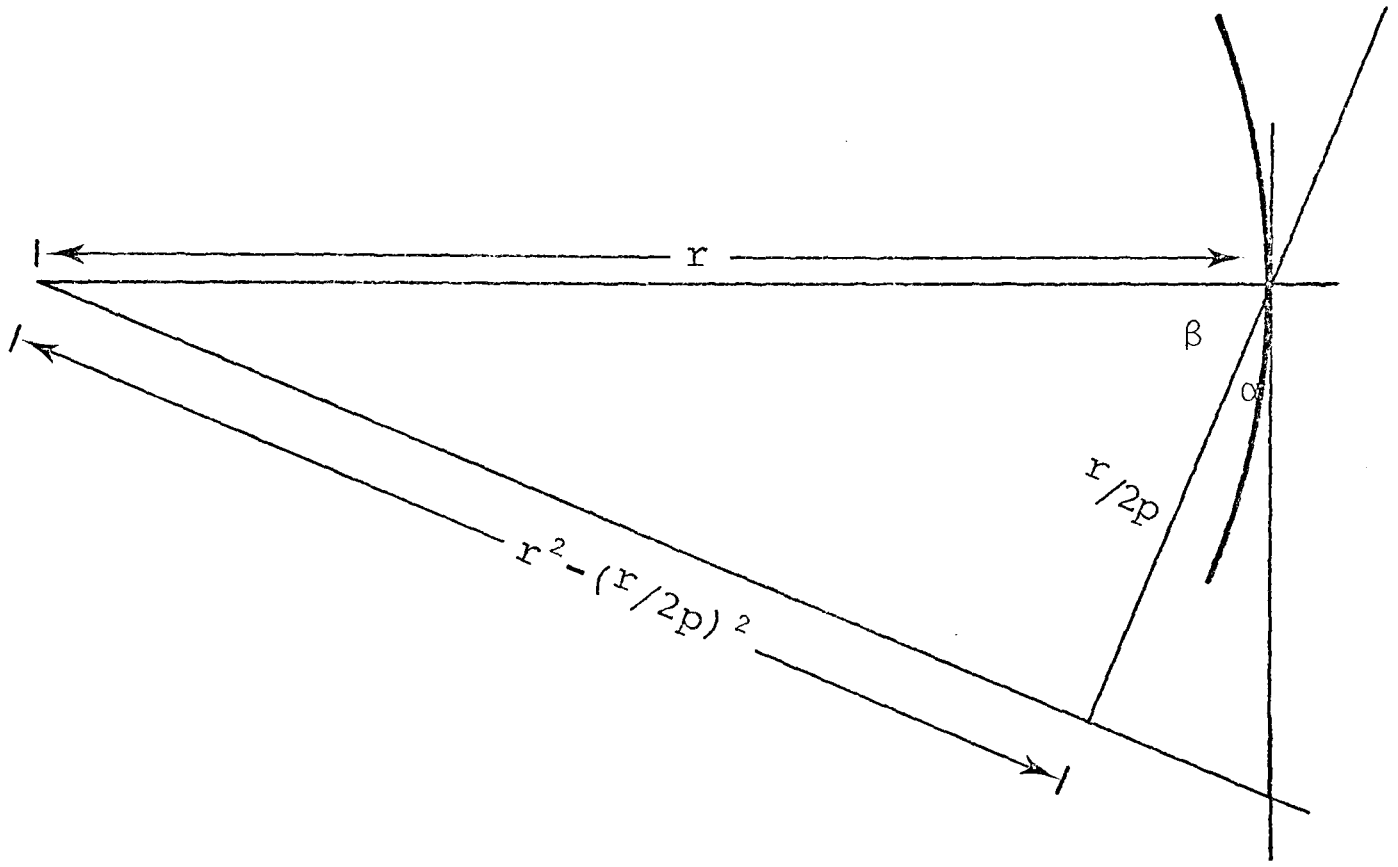
N = Number of plasmodesmata per µm of cell wall

then $F = \frac{N}{\text{sample thickness}}$ 3.

Thus for a section of thickness = T:



The sample thickness = $(T + 2\nu)$ where ν is dependent on the value r/p, the fractional value of r which is the minimum arc of section at which a plasmodesma may still be visualized. The value of p is dependent on the minimum angle (α) at which the membrane lining the plasmodesma may be sectioned and still resolved. These two parameters are related as follows:



$$\alpha = 90 - \beta$$

$$\sin \beta = \frac{r^2 - (r/2p)^2}{r^2}$$

$$= 1 - {}^{1}/_{4p^2} \qquad 4.$$

When $\alpha + \beta = 90$

$$\cos \alpha = \sin \beta$$

$$\text{hence } \cos \alpha = 1 - {}^{1}/_{4p^2} \qquad 5.$$

For angles $<\alpha$ the tubule becomes invisible.

Gunning has proposed (Robards, 1976 : p.54) that if any

portion of the plasmodesma is detectable then

$$F = \frac{N}{(T + 2r)} \qquad 6.$$

but points out that this is impossible. He then erroneously assumes that if half the radius is visible then

$$F = \frac{N}{(T + r)} \qquad 7.$$

and further that if one quarter of the radius is present then

$$F = \frac{N}{(T + 1.5r)} \qquad 8.$$

In fact, $\nu^2 = r^2 - (\frac{1}{2}\ r/p)^2$ 9.

and thus $\nu = r\ \sqrt{(1 - 1/4p^2)}$ 10.

where r/p is the fraction of r that is considered to be the minimum arc of section that may be resolved.

Thus:

$$F = \frac{N}{T + 2\nu} = \frac{N}{T + 2r\ \sqrt{(1 - 1/4p^2)}} \qquad 11.$$

As $r/p \to o$ then $\nu \to r$ and equation 11 → equation 6.

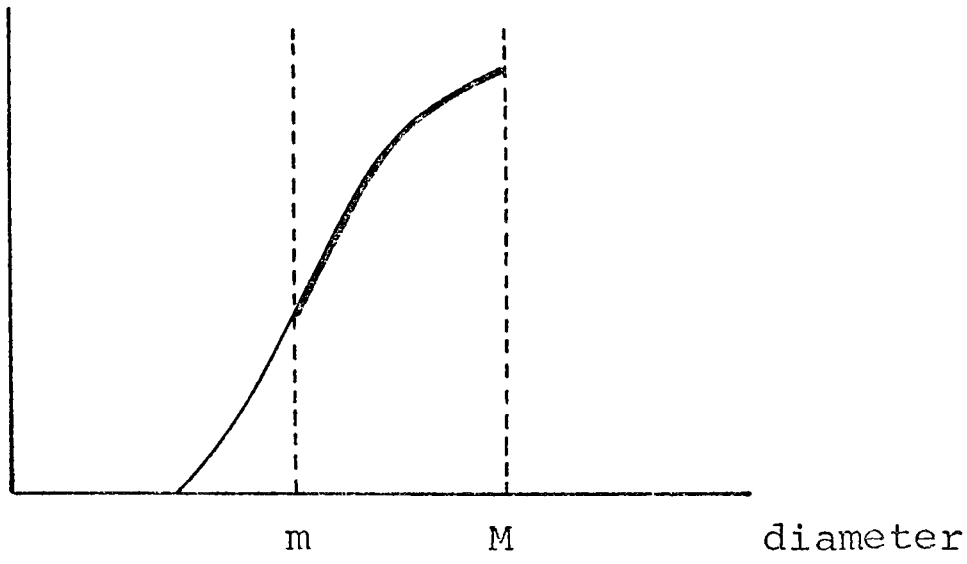
But when $p = 2$, $F = \frac{N}{(T + 1.94r)}$ 12.

and when $p = 4$, $F = \frac{N}{(T + 1.98r)}$ 13.

showing that equations 7 and 8 are substantially incorrect.

Can we estimate the value of r/p required? If $p = 4$, which Gunning (Robards, 1976) considered a reasonable resolution, then the angle of section of the membrane, α, = 10^{o} 10' which is excessively acute. What is required

is a measure of α. This may be obtained by measuring the dimensions of large numbers of sections through a uniform series of plasmodesmata from a single tissue. The distribution of these will fall onto half of a normal curve.



The maximum value (M) will give the mean diameter (2r) whereas the minimum value (m) will provide a value for $^{2r}/_{p}$ from which ν may be calculated using equations 9 and 10 and substituting into equation 11.

The value of T required must be estimated from the interference colour of the section by the method of Peachey (1958).

Figure 7.1 Transverse section through a digestive gland which has been stimulated for three days with 4%(w/v) bactopeptone and then treated for 2 h with stimulant solution containing 100 mM-sodium chloride. Note how the core material of the labyrinthine walls and the periphery of the vacuoles are particularly heavily stained. Also note the lack of deposits in the endocuticle and the lipid droplets of the stalk cell.

Stimulated tissue - 3 days: fixed glutaraldehyde + silver I nitrate: Spurr's resin: no poststain. x 8,430.

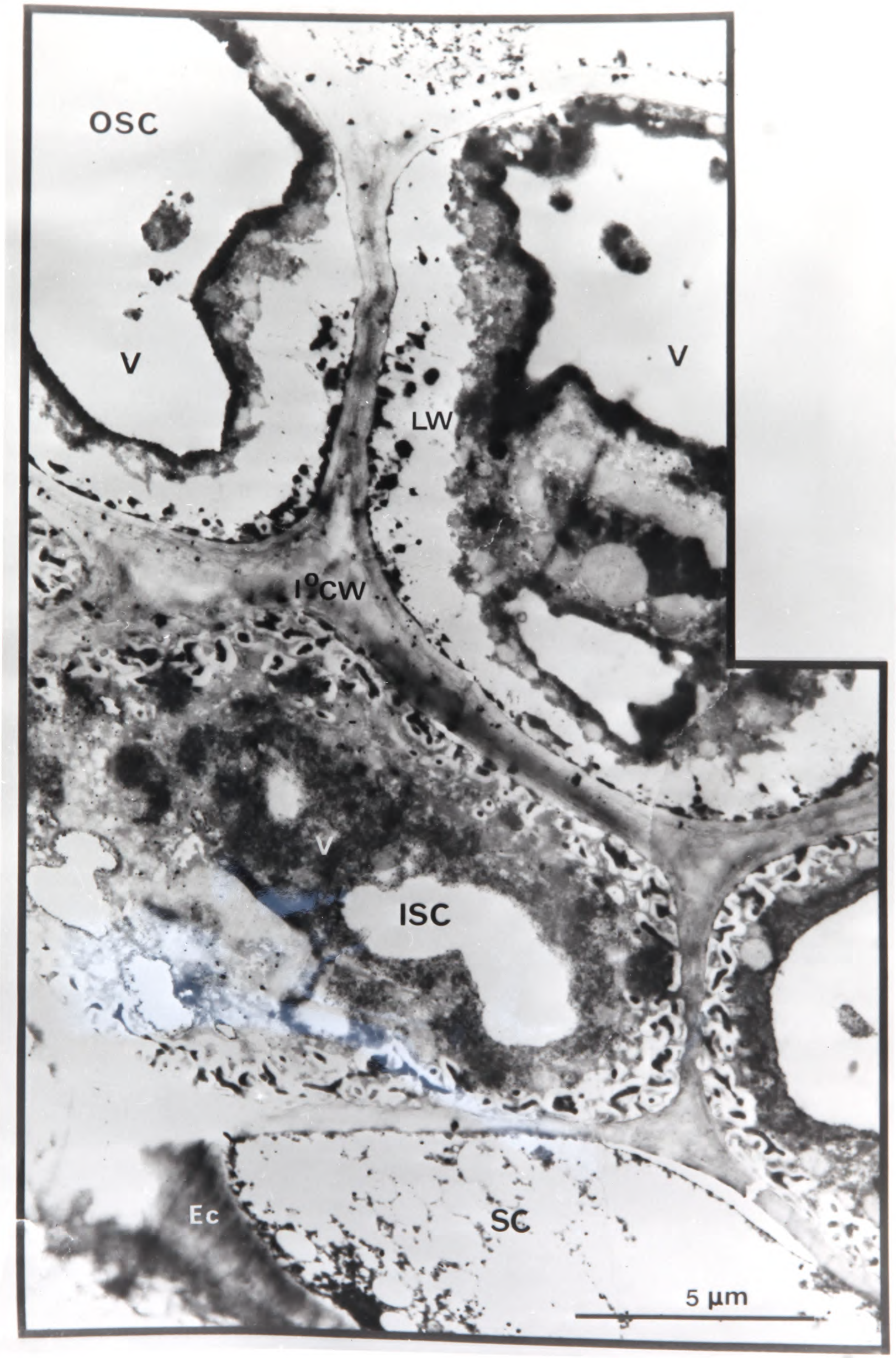

OSC
V
V
LW
I°CW
V
ISC
Ec
SC
5 µm

Figure 7.2 Detail from Fig. 7.1 showing the fibrillar staining of the core material in the labyrinthine cell wall. Note the similarity in staining of the primary cell wall to tissue treated with mercury I chloride - bromophenol blue (Figs. 4.13 - 4.15).

Stimulated tissue - day 3: preparation as Fig. 7.1.
x 24,580.

Figure 7.3 High-voltage electron micrograph of a thick section showing the cell wall between several gland cells in tissue prepared from traps fed for three days with 4%(w/v) bactopeptone containing 100 mM-sodium chloride. Note the presence of deposits of silver I chloride in the vacuole and along the plasmalemma.

Stimulated tissue - day 3: fixed osmium tetroxide + silver I nitrate: Spurr's resin: lead citrate poststain.
x 16,130.

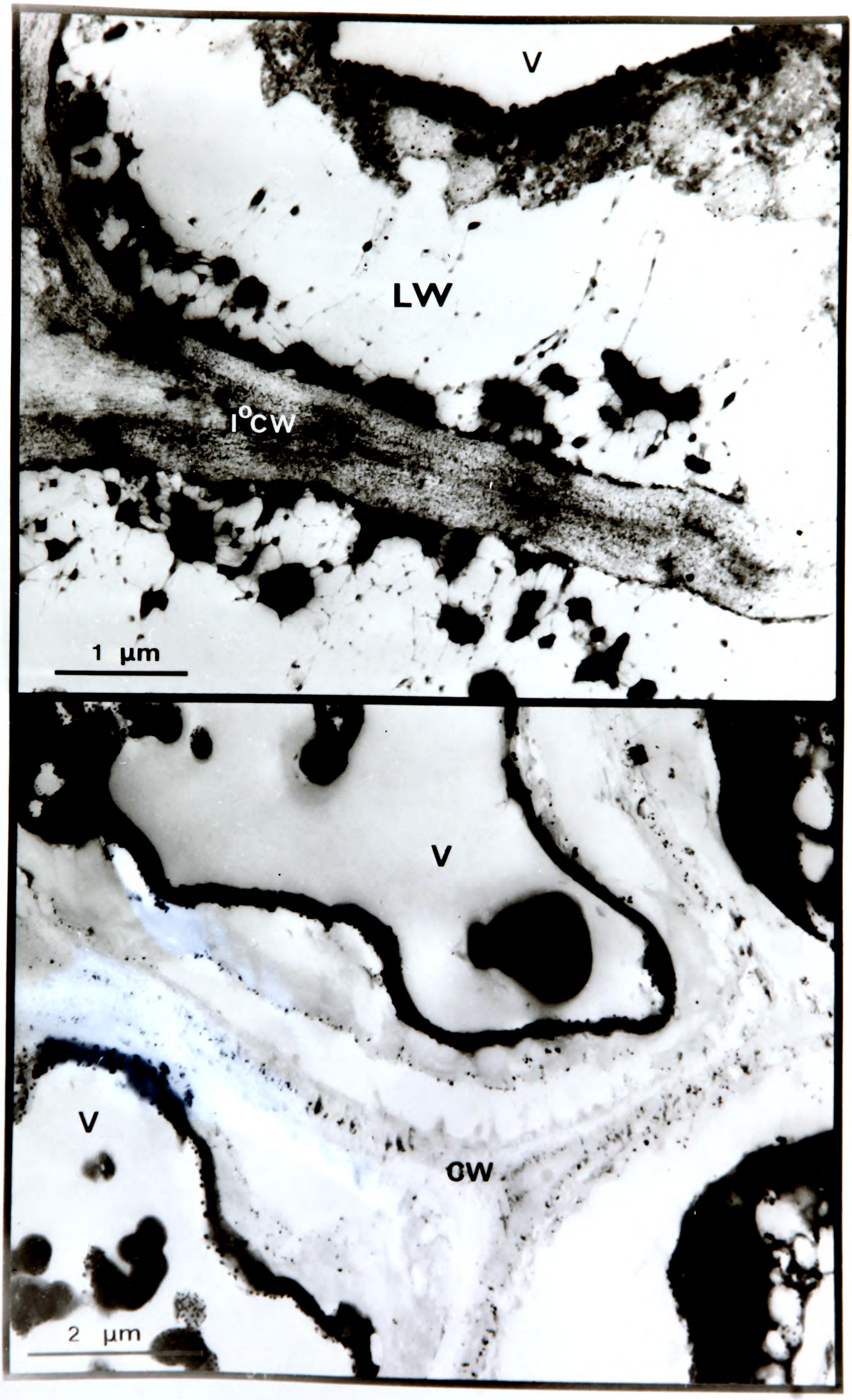

V
LW
I°CW
1 µm
V
V
OW
2 µm

Figure 7.4 Part of the outer layer of secretory cells in a digestive gland treated as in Fig. 7.3, but seen in thin section. Note the heavy deposits of silver I chloride along the plasmalemma, the highly amoeboid vacuoles, and the dense deposits along the external cell wall.

Stimulated tissue - day 3: fixed osmium tetroxide + silver I nitrate: Spurr's resin: no poststain. x 6,080

Figure 7.5 Detail from an outer secretory cell showing how parts of the cytoplasm appear to have become engulfed by the vacuole as a result of the chloride treatment. Control section treated as in Fig. 7.3 but fixed without silver I nitrate.

Stimulated tissue - day 3: fixed osmium tetroxide: Spurr's resin: lead citrate poststain. x 12,960.

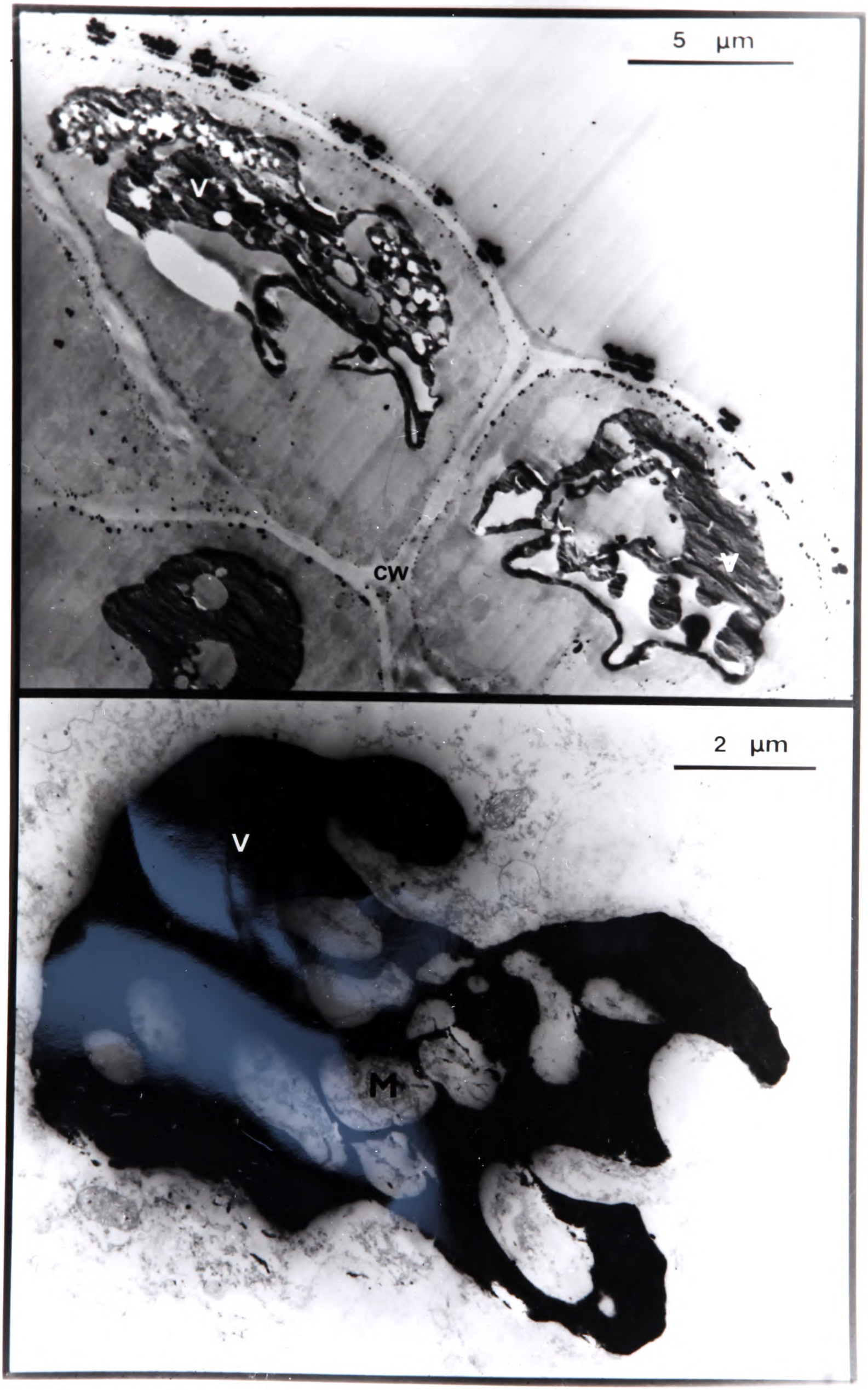

5 µm
V
CW
V
2 µm
V
M

Figure 7.6 Transverse section showing part of a gland treated as in Fig. 7.3. Note how the deposits of silver I chloride occur particularly densely along the edges of the stalk cell and within the vacuoles.

Stimulated tissue - day 3: fixed glutaraldehyde + silver I nitrate: Spurr's resin: lead citrate poststain. x 3,100.

Figure 7.7 Detail from Fig. 7.6 showing the endocuticle. Note the heavy deposits within the stalk cell abutting the endocuticle compared with the few grains within the wall.

Stimulated tissue - day 3: as Fig. 7.6. x 16,140.

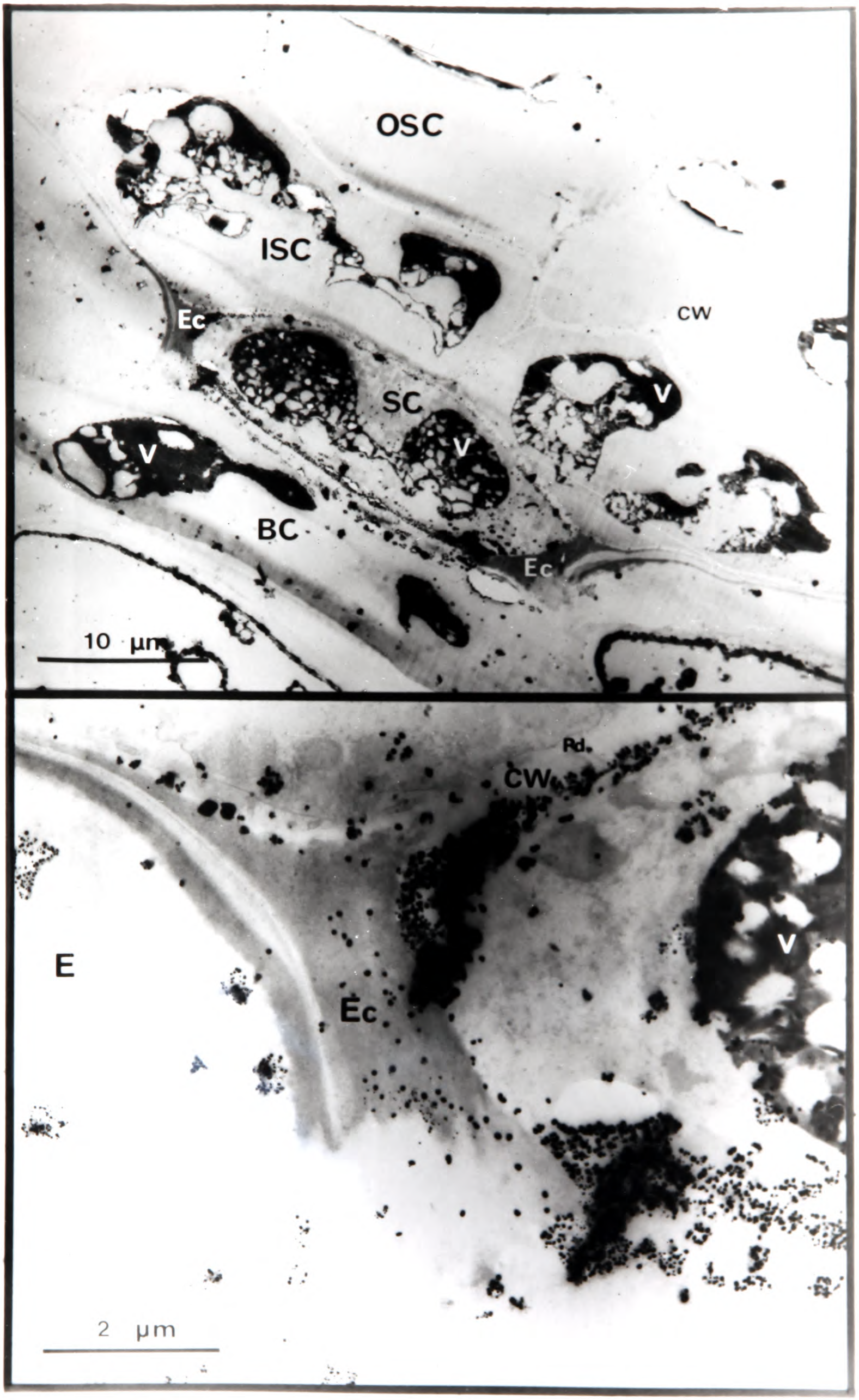

OSC
ISC
Ec
CW
SC
V
V
V
BC
Ec
10 µm
Rd.
CW
V
E
Ec
2 µm

Figure 7.8 Detail from the cell wall between the stalk cell and the basal cell of a gland from tissue prepared as in Fig. 7.3. Note how the silver I chloride deposits are confined to the plasmodesmata.

Stimulated tissue - day 3: fixed osmium tetroxide + silver I nitrate: Spurr's resin: no poststain.
x 21,220.

Figure 7.9 Detail from the cell wall between the stalk cell and basal cell of a gland from tissue prepared as in Fig. 7.3. Note how there is a heavy deposit of silver I chloride along the edge of the wall but only crossing the wall in the pit-field.

Stimulated tissue - day 3: fixed osmium tetroxide + silver I nitrate: Spurr's resin: lead citrate poststain.
x 13,540.

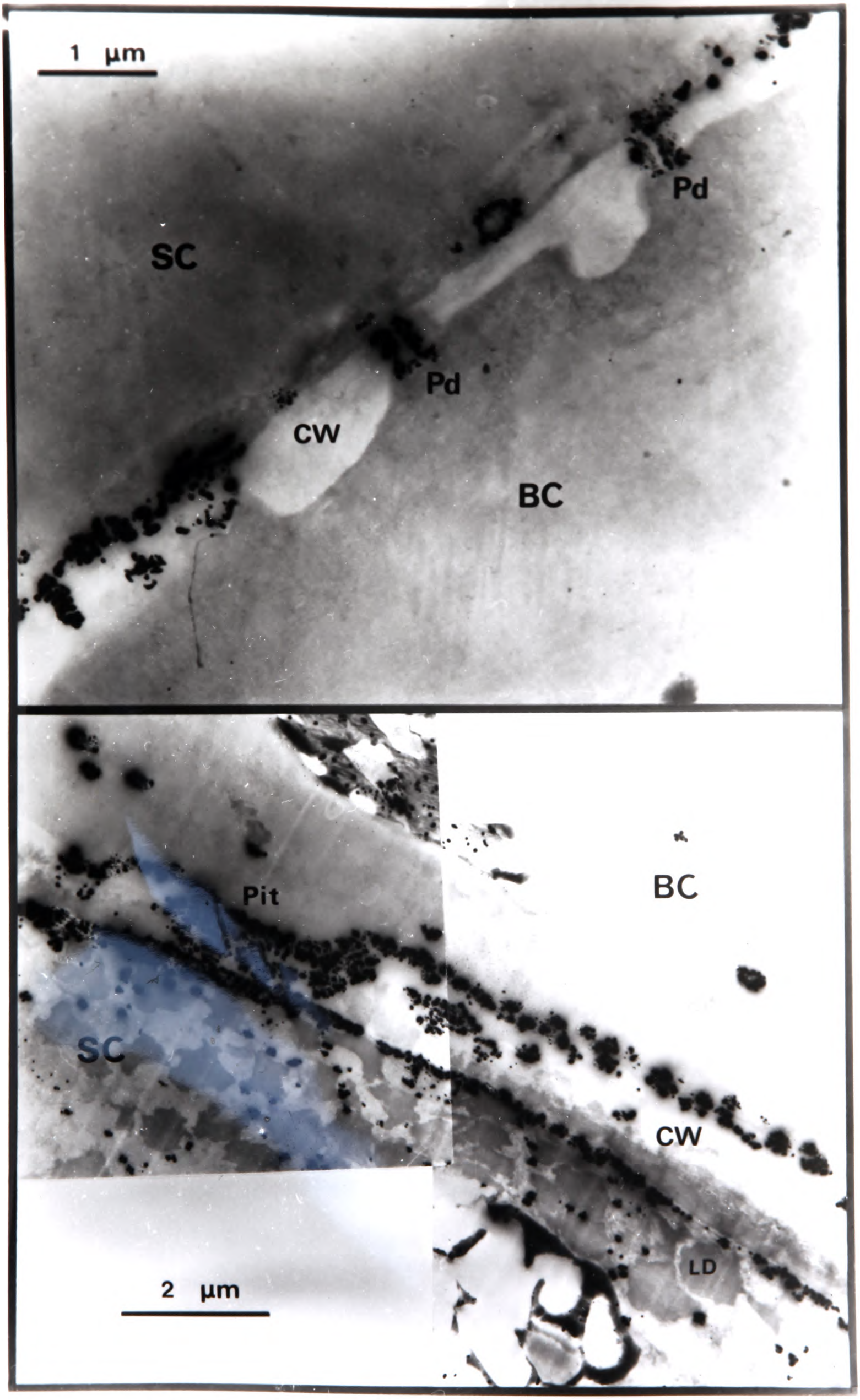

1 µm
SC
Pd
Pd
CW
BC
Pit
BC
SC
CW
LD
2 µm

Figure 7.10

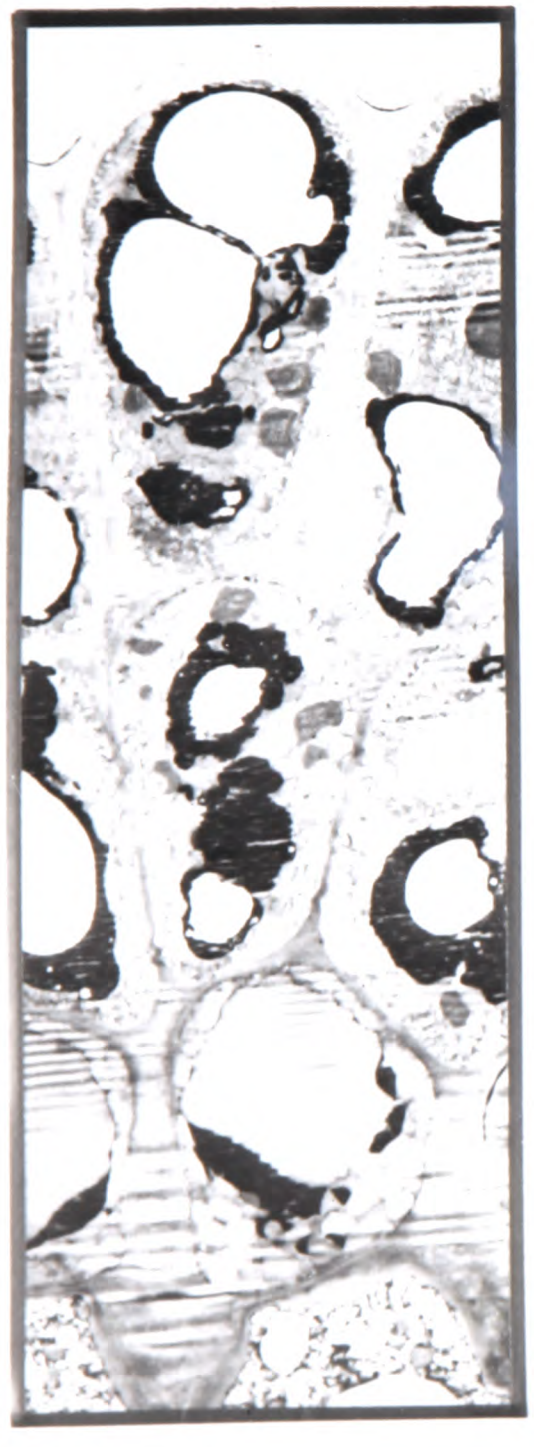
a) Showing the area of each of the boundaries within the gland

● area of boundary

○ area of labyrinthine wall

b) Showing the relationship between the amplification factors at each boundary within the gland

c) A cross section of a gland showing the spatial relationship between the different boundaries given in a) and b). Outer secretory cell to left, stalk cell to right. x 2,770.



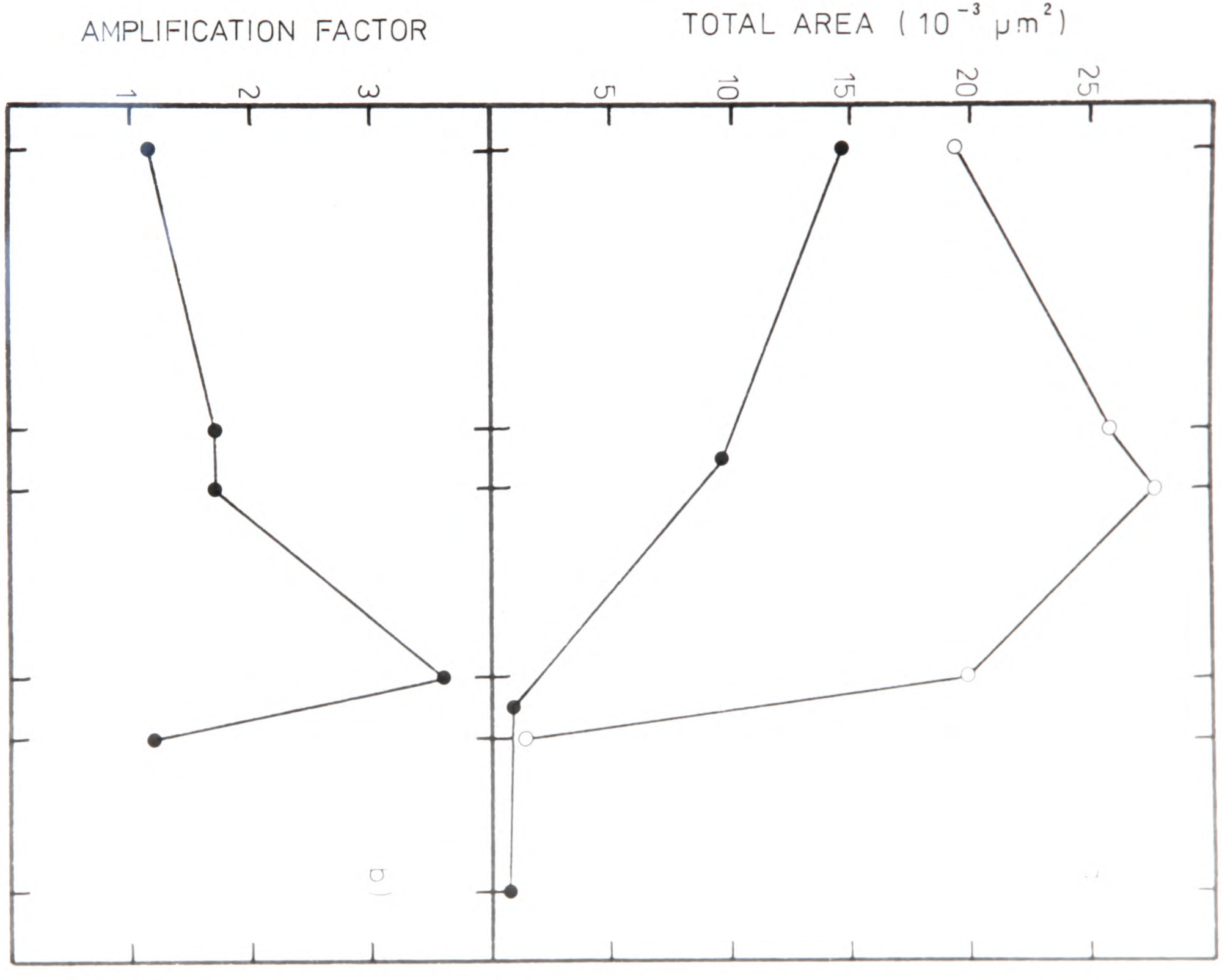
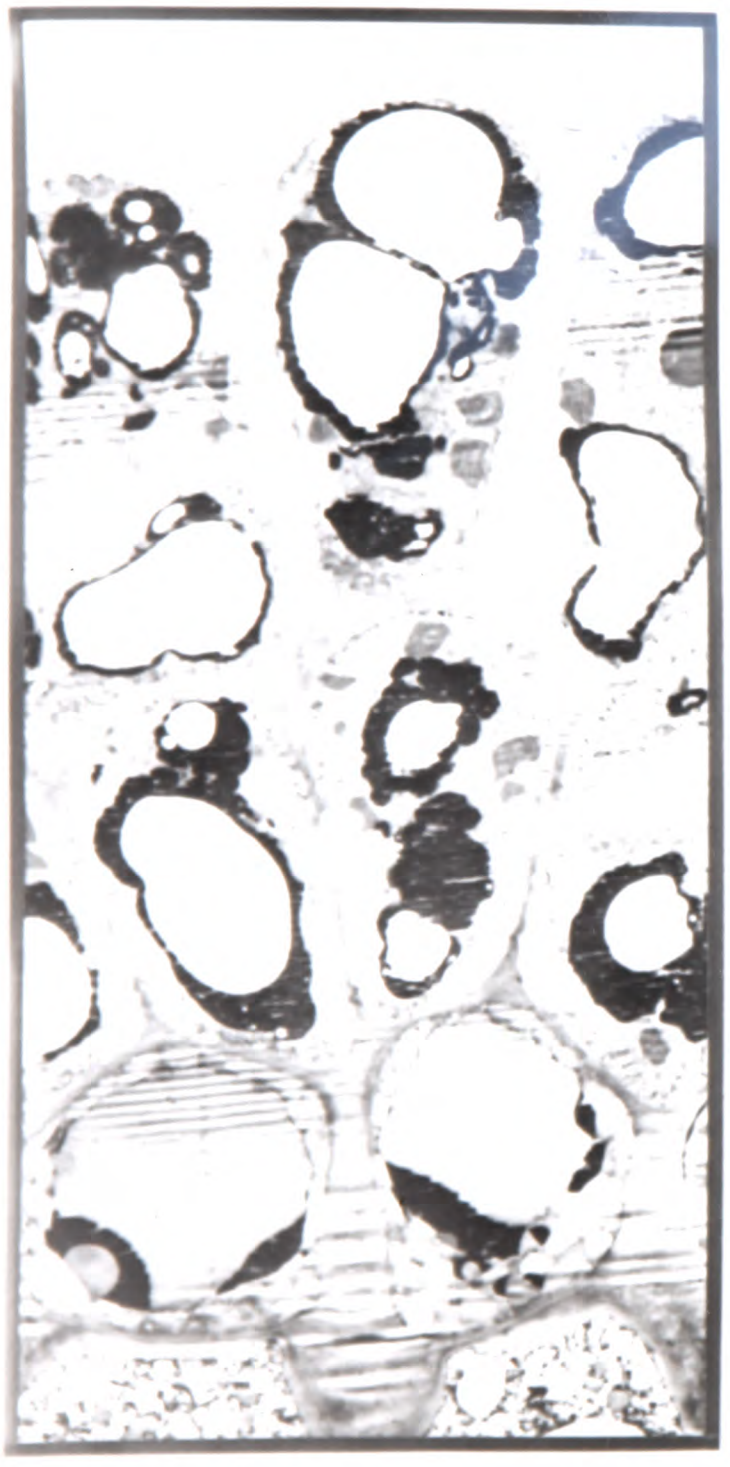

AMPLIFICATION FACTOR
1
2
3
TOTAL AREA (10^{-3} μm^2)
5
10
15
20
25

Figure 7.11 Showing the relationship between the plasmodesmata at each boundary of the gland.

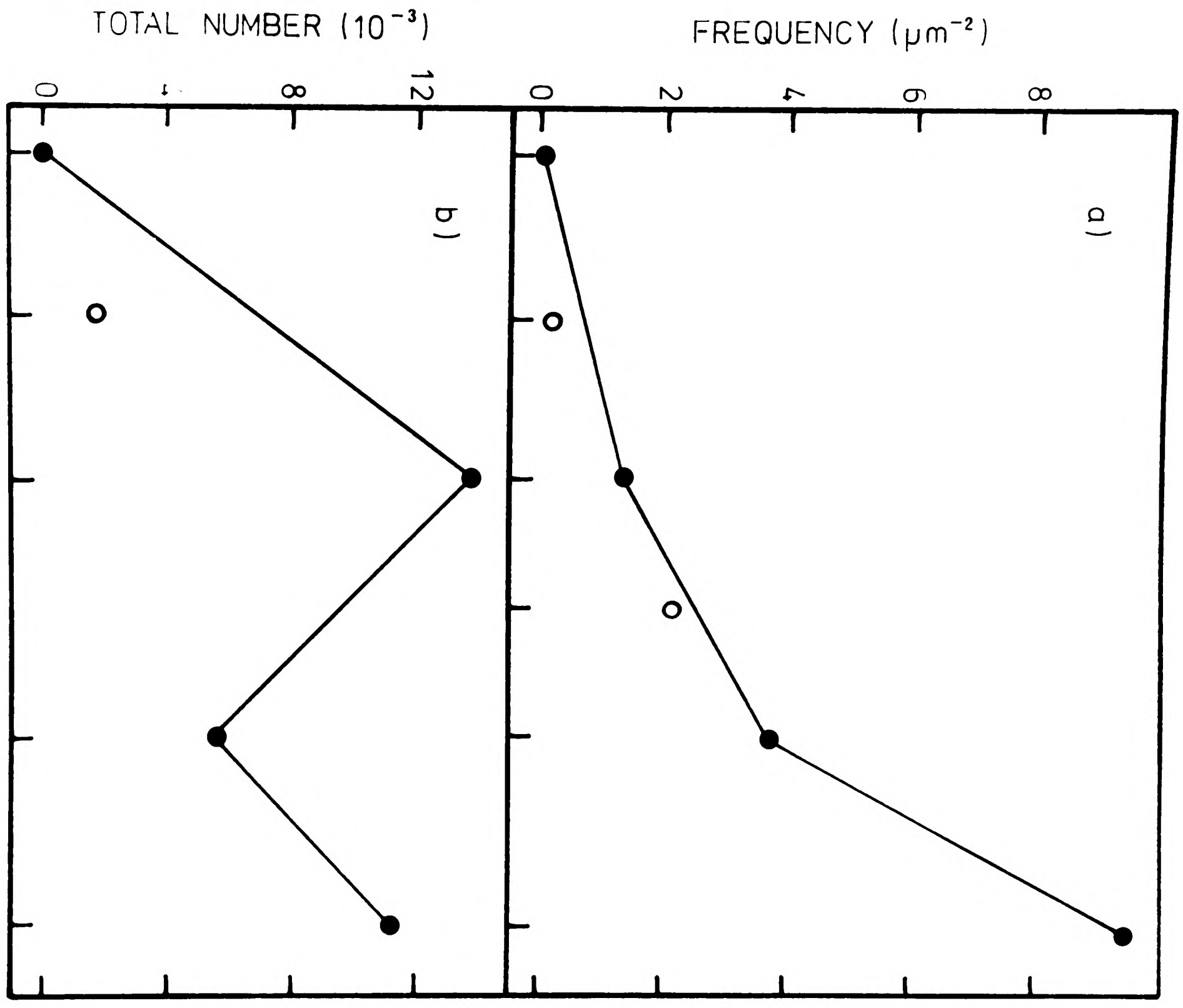
a) Number of plasmodesmata per μm^2

b) Total number of plasmodesmata in that boundary

c) A cross-section of a gland showing the spatial relationship between the different boundaries given in a) and b). Outer secretory cell to left, stalk cell to right. x 2,770.



PLASMODESMATA



CHAPTER 8

CONCLUSIONS

"Was not the first animal that ever lived,
a plant that found out the blessing of a
stomach and ran away with it?"

Burnett (1829)

A wide variety of glands exist in numerous species of plants. These usually have either an absorptive or a secretory function but in the carnivorous plants many of the glands clearly perform both these functions. The substances secreted may be hydrophilic or lipophilic but, at least in exotropic glands, the overall structure is often rather similar, with a cap of secretory cells, a stalk which has a suberized or cutinized cell wall and a basal cell embedded in the epidermis. In some cases, such as in Drosera and Drosophyllum, this structure may become modified by the gland being supported on a long stalk containing vascular tissue but the overall structure of the functional part of the gland is unaltered.

Structurally, the stalked glands of Drosera are the most complex plant glands known, but functionally Dionaea is the most complicated system yet examined in detail, showing extreme sophistication in some aspects of its activity. Complex sub-structural and biochemical features have evolved, which enable the plant to obtain the maximum amount of nutrient for the minimum amount of effort.

The first point at which economy of effort is apparent occurs immediately after the trap has closed but before any secretion has been stimulated. The plant is clearly capable of distinguishing 'useful' from 'non-useful' matter and will only secrete when the former is present (Darwin, 1875). This aspect is, however, even further refined. Initially, when the trap shuts the closure is incomplete, adopting the 'closing' position (Ashida, 1934) seen in the

right-hand trap of Fig. 1.1. Jones (1923) found that the size of prey held within secreting lobes of Dionaea had a minimum size of 5 - 6 mm whereas the majority of arthropods seen moving over the leaves were a small species of ant, 2 - 3 mm in length. If traps were made to close over these ants, the insects easily escaped through the spaces between the marginal spikes. He thus confirmed, in the field, Darwin's conclusion that "it would manifestly be a great disadvantage to the plant to waste many days in remaining clasped over a minute insect and several additional days or weeks in afterwards recovering its sensibility; inasmuch as a minute insect would afford but little nutriment. It would be far better for the plant to wait for a time until a moderately large insect was captured, and to allow the little ones to escape" (Darwin, 1875). Hence, as has been appreciated for some time, the trapping mechanism is designed to prey selectively upon the arthropod population.

Once a suitable-sized prey is captured, secretion will commence. Darwin (1875) suggested this to be due to nitrogenous matter being absorbed by the trap and this has since been confirmed (Robins, 1975; 1976). Furthermore, the trap has developed a greater sensitivity to some compounds than to others, those that act as the most effective secretagogues being compounds which are likely to be present within the prey (Robins, 1975; 1976). Lichtner and Williams (1977) have claimed to stimulate secretion purely by mechanical agitation of the trigger hairs but there are reasons, discussed earlier, to suppose that this is not the digestive fluid. It is possible, however, that they are

observing a physiological effect. Perhaps the death-throes of the captured prey, which will agitate the trigger hairs, stimulate only the mechanism for the release of fluid from the gland. This will bathe the struggling prey, washing off nitrogenous molecules which come into contact with the leaf surface and on entering the glands, stimulate hydrolase release by some as yet unknown mechanism.

This simple model fits most of the observations made on the activity of these glands. There are, however, enzymes apparently stored within the gland-head cell walls and it is difficult to envisage how these would not be lost during the initial flush of fluid from the gland. A better understanding of how fluid is released may help to overcome this problem.

Once stimulated, fluid containing digestive enzymes is freely released provided that stimulation is continued. Darwin (1875) found that if the stimulant was removed from a secreting trap then the trap ceased to secrete and re-opened. This phenomenon is presumably comparable to the trap reaching the end of a normal cycle and re-opening, ready to capture further prey. The trap will not, however, continue to secrete indefinitely. When supplied daily, as here, with fresh stimulant solution the rate of secretion is linear for 6 - 8 days then diminishes despite continued stimulation. This is, I believe, a physiological effect and not due to the design of the experiment. Canby, in correspondence with Darwin (Jones, 1923), wrote that he found leaves did not function again if they had a very

large meal but would function several times when smaller prey was caught. Both Darwin (1875) and Balfour (1875) also observed this and noted that meat fed to the leaves would only putrefy if it was so large as to prevent the lobes closing properly. The size of the lobe, then, defines the maximum size-limit of the meal. Thus, if presented with a very large prey, it is to the plant's disadvantage to continue to secrete after 6 - 8 days as the prey is likely to become seriously contaminated with micro-organisms which will deprive the plant of the nutrients it is trying to acquire, or even infect the digestive glands. In addition, each plant presumably has a limit to the amount of nitrogen which can be assimilated in a short period of time and a large meal may exceed this limit. Under these circumstances, it will be as much to the plant's advantage to cease to secrete, even though chemical stimulation is continued, as it is not to begin secreting until a suitable prey is caught.

The evidence presented here shows that, like other genera of carnivorous plant, Dionaea has hydrolytic enzymes stored within the glands prior to stimulation. It appears that they are stored within the small osmiophilic vacuoles, the smooth endoplasmic reticulum and the cell walls of the two outer layers of gland cells. The way in which stimulation initiates their discharge is yet to be discovered but there seems to be a rapid initial release from the intracellular sites of storage into the cell walls, considerably depleting these sites of their protein. This appears to take place at least in part by the endoplasmic

reticulum fusing to the cell wall at the labyrinthine elaborations and releasing the material from the cisternae directly into the extracellular space. In addition, discharge may occur by vesicles, derived from the endoplasmic reticulum migrating to the cell periphery and fusing with the plasmalemma. Such modes of discharge are not unprecedented but are certainly unusual, the more common pathway being a movement of vesicles from the endoplasmic reticulum to the dictyosomes then via dictyosomal vesicles to the cell periphery. It is suggested that there is no need for an involvement of dictyosomes in Dionaea as a carbohydrate component, shown to be supplied to the secretion by the dictyosomes in many other secretory systems, is not present here.

Instead, the dictyosomes are proposed to be concerned with the construction of the labyrinthine walls. Although a little evidence for this was obtained in some electron micrographs, the proposal needs to be tested by examining the activity of the dictyosomes during the maturation of the glands. The labyrinthine wall is apparently laid down during the last few days of maturation. Hence, if supplying material for this is the primary role of the dictyosomes, greatly increased activity might be expected during this phase. An alternative possibility is that the dictyosomes are involved in the supply of fluid for the secretion, such as occurs in Monarda fistulosa (Heinrich, 1973a, b). The total lack of any observed stimulus-induced alteration in the dictyosomes lends no support to this alternative, although there could be a burst of activity within a few

hours of stimulation which is dissipated within the first two days.

There is a considerable body of evidence in the literature to support a close association between endoplasmic reticulum and vacuoles and for the storage of protein within vacuoles. Presumably, then, in Dionaea the protein in the vacuoles of the secretory cells is made on the endoplasmic reticulum as the gland develops and the vacuoles form from this. A microautoradiographic study of this process would serve to clarify this point by pulse-chase experiments with tritiated leucine, if sufficient radiolabel could be introduced in a single dose. This was found to be a problem here and even after long exposures to large amounts of radiolabel, the grain counts were very low. Sufficient counts were obtained, however, to give an indication of the pathway of discharge.

In other species of carnivorous plant there is no evidence to suggest that once a digestive gland has been stimulated it is able to function again. In Pinguicula, Heslop-Harrison (1976) found the sessile glands to degenerate after performing a single secretion and absorption cycle. These glands contain large concentrations of hydrolases prior to stimulation which are rapidly lost into the fluid pool. What makes Dionaea possibly unique is that, as the trap can undergo a further cycle of activity, the intra-glandular stores of enzymes destined for secretion must be recharged. This could occur either during the current cycle of activity or after it is completed. The former period has the advantage that a plentiful supply of

small molecular precursors for protein synthesis are present, as these are being absorbed by the gland from the exterior. Further, there would be a much shorter lag-period after the completion of one cycle before the next could begin.

Evidence from both ultrastructural and biochemical observations is presented which shows that, following stimulation, further enzymes are synthesized de novo within the gland while the trap is still undergoing active secretion. Some of these enzymes are secreted while others, possibly, are moved into an intracellular store within the small vacuoles.

It appears, then, that Dionaea has two lines of attack. As with other carnivorous species, there is an initial rapid discharge of stored hydrolases in response to chemical stimulation. Subsequently, however, Dionaea uses some of the freshly-absorbed nutrients to make more digestive enzymes which are released from the secretory cells, in addition to the stored enzymes. This apparently continues until, in some way, the gland ceases to respond to stimulation and secretion is terminated. At no point in the cycle does all the secreted protein appear to be synthesized de novo. Both the inhibitors of protein synthesis exhibited, deuterium oxide and cycloheximide, cause a marked decrease in the digestive activity of the secretion, but do not completely destroy the discharge of hydrolases from the glands. Presumably, as the digestive cycle progresses, the proportion of newly-synthesized hydrolases stored increases so that when secretion ceases altogether, the glands are

left fully charged. The slow increase in the number of silver grains in the small vacuoles after 3 - 4 days of activity supports the suggestion that storage of new enzymes may start early on in the cycle.

Thus with the production and utilisation of its digestive enzymes, as with the mechanism for the capture of prey, Dionaea seems to make the maximum use of the resources available. The great majority of the nitrogen, and probably most other nutrients, needed by the plant are obtained from trapping prey. It was shown by Oxford Scientific Films, using time-lapse photography, that resting plants grow extremely slowly but that when prey is caught a spurt of rapid growth occurs. This suggests that the pool of readily-available nitrogen within the plant is very restricted and that growth and reproduction are dependent on capturing prey. The system adopted, using a small store of hydrolase activity followed by synthesis of further digestive enzymes as required, means that the minimum proportion of the nitrogen within the plant is committed to the essential store of hydrolytic enzymes, leaving the remainder available for other uses.

Why, then, have an intracellular store at all? The trap is so designed that once a suitable-sized prey is caught it cannot escape and therefore a mechanism in which the presence of prey only stimulated de novo protein synthesis would, perhaps, suffice. This, however, requires a store of precursor molecules for protein synthesis and it appears to be more advantageous for plants to store completed proteins rather than the component amino acids as

has been found, for example, in seeds. Furthermore, competition between plants will tend to favour those that most rapidly assimilate digestive products and can thus grow larger traps (to catch larger prey) and the mechanism which has evolved may be the best balance between storage and synthesis needed to achieve this.

The package of hydrolases released by the glands appears to be well-suited to its task. Containing several peptide hydrolase activities, it can presumably reduce most of the protein in the prey to a form in which it can be taken into the glands. A more detailed examination of this peptide hydrolase activity is needed. Although it has been shown that both endo- and exo-peptidases are present, little detail is known about their activity. Moreover, nothing is known about the mechanism of their storage. For example, they may be inactivated in some way - such as by being held under alkaline conditions - or possibly they are stored as proto-enzymes, requiring proteolytic cleavage to become active, as with the pancreatic system. In addition to the proteases, the secretion contains nuclease activity which will degrade nucleic acids, providing a further significant source of nitrogen for the plant.

The chitinase activity in the secretion also serves to increase the amount of nitrogen derived from the prey by attacking the exoskeleton. The level of this activity, 1.64 pkat(mg protein)$^{-1}$, is sufficient to produce about 130 µg NAG day^{-1} in an average-sized trap or to hydrolyse about 1 mg of chitin within a digestive cycle. In an insect like Calliphora, which weighs about 60 mg (wet weight), this

means that if chitin forms about 1 - 2% (w/w) of the insect then there is sufficient activity in the secretion for it to be entirely digested. The whole exoskeleton is not, however, completely degraded but it is not known how much of the undigested material is chitin.

The presence of high levels of acid phosphatase activity in the secretion is initially puzzling, particularly if, as Scala et al (1969) found, it has no sugar phosphatase activity. For the latter enzyme to be a component of the secretion is quite reasonable as it would convert a number of metabolites within the prey into a form in which they could be more readily absorbed. Yet despite considerable activity against p-Nitrophenyl phosphate as a substrate, the secretion appears inactive against several hexose phosphates. A similar situation was found with the secretion of Drosera by Clancy and Coffey (1977). p-Nitrophenyl phosphate is, however, an ester and therefore any enzymes present with suitable esterase activity may be able to attack this substrate. Many peptide hydrolases can act as esterases - indeed the specific substrates used to analyse particular types of proteolytic activity are often esters of suitable structure. Similarly glycosidases, such as chitinase, may be analysed by using their esterase activity. Furthermore, the secretions of Dionaea (Scala et al, 1969) and Drosera (Clancy and Coffey, 1977) both show considerable activity against nucleoside triphosphates and the same enzyme will almost certainly hydrolyse p-Nitrophenyl phosphate. Scala et al (1969) concluded that "... in consideration of the role of the plant to trap, kill, and digest

insects it [that the fluid contains so much phosphatase activity] is not surprising; since destruction of ATP in the insect would certainly reduce its ability to escape entrapment...." (Scala et al, 1969). This seems, however, extremely unlikely as prey of suitable size cannot escape once the trap is sprung while if it is too large (or small) it will have escaped long before secretion begins. In fact, captured prey tends to struggle ceaselessly until overcome by exhaustion, which also happens long before secretion begins, and very little ATP will remain within an insect's body at exhaustion. Hence the most likely explanation seems to be that the majority of the hydrolytic activity against p-Nitrophenyl phosphate is an expression of the esterase activity of peptide hydrolases, glycosidases, and anhydrases and is not in fact due to a specific phosphoric monoester hydrolase at all. Thus the hydrolytic package released from Dionaea digestive glands is well adapted to obtaining the maximum amount of nutrient from the prey.

The final feature required to complete the digestive activity of the trap is an ability to absorb the products of digestion. That this occurs in Dionaea simultaneously with the secretion of digestive enzymes shows a degree of organisation far greater than apparently found in Pinguicula (Heslop-Harrison, 1975; 1976), the only other genus for which a mechanism for the absorption of the organic products of digestion has been proposed. Although Lüttge (1964b) showed the glands of Nepenthes pitchers to absorb L-[^{14}C]-leucine, he was principally interested in the movement of inorganic ions in and out of the glands. His findings are summarized

in Lüttge (1971). While it is possible that in carnivorous plants the absorption of organic nutrients involves co-transport of inorganic ions, there is no direct information available on this. In their experiments on the uptake of calcium from milk, Gilchrist and Juniper (1976) proposed that the calcium was migrating with whole or partially degraded milk-protein and that this was why it remained in the tissue during fixation and embedding. They noted that the rate of transport was higher than expected for the diffusion of free calcium ions across the gland (Juniper, personal communication), suggesting that transport of the calcium was being accelerated because it was associated with the protein.

The mechanism proposed by Heslop-Harrison (1976) that both secretion and absorption in Pinguicula are determined by the mass flow of water within the gland is, as she points out, clearly unsuitable as an explanation of the activity in Dionaea, where a bi-directional flow of metabolites is taking place. A mechanism of absorption is put forward here whereby this bi-directional flow may be maintained. It is proposed that the material being absorbed is taken into the cells and moved through the symplast, while the secretory enzymes are discharged into the apoplast directly from the endoplasmic reticulum. Once in the extracellular space, these enzymes move out of the apoplast, passing through the thin cuticle to the exterior. This may occur simply by diffusion but some system by which they were flushed out of the gland by fluid movement would be more satisfactory.

It has been suggested in Chapter 7 that the highly

elaborated labyrinthine walls of the inner secretory cells function to provide an extremely active surface of absorption and thus create a concentration gradient of digestive products into the gland. It is now hypothesized that they also excrete fluid in sufficient quantities to create a strong positive water potential across the gland. As there is no evidence for reversed pinocytosis within the secretory cells, it is unlikely that this mechanism is involved in water excretion, as it appears to be in Monarda fistulosa (Heinrich, 1973a, b). It is therefore probable that water transport is caused by establishing electrical or ionic gradients across the plasma membranes of the secretory cells. This hypothesis is open to investigation by the conventional techniques of electro-physiology. The pH of the medium in the lumen of the trap is acidic, with reports of acidity as high as pH1.5 (Lichtner and Williams, 1977). This tends to suggest that hydrogen ions are being excreted, possibly as counter-ions to actively excreted chloride ions. Morrissey (1955) found a marked rise in the chloride ion concentration of Nepenthes pitchers at maturation, from "very low" to 6 - 9 mg ml^{-1}, while the pH simultaneously dropped from pH5.5 to 3.3. If this suggestion proves to have some foundation in Dionaea then once again the plant is employing a mechanism which displays great economy of effort, as it would be using one operation to achieve two results.

Such a mechanism would, however, prove futile if there was apoplastic continuity between the main tissue of the leaf and the secretory cells of the gland. Such continuity

is prevented by the endocuticle (see 3:II:2). It is often assumed that this impregnated wall serves to prevent excess water loss from the leaf by transpiration through the glands but Dionaea grows in boggy habitats where water shortage is seldom a problem. Instead, the primary function of the endocuticle in Dionaea seems to be to enable the plant to establish a positive water potential across the gland, the maintenance of this water potential being one of the two main functions of the extended plasmalemma of the inner secretory cells.

Hence, the models proposed for the simultaneous secretion of hydrolytic enzymes, excretion of fluid, and absorption of the products of digestion are compatible one with another. They are also compatible with the following points:

a) The observed ultrastructure of the secretory cells and the dynamic changes in the ultrastructure following stimulation.

b) The suggestion from previous work (Robins, 1975; 1976) that the release of protein and the release of fluid may be under separate control mechanisms.

c) The induction of the secretion of a low pH fluid (pH1.5 - 3.5) by continuous agitation of the trigger hairs (Lichtner and Williams, 1977).

With reference to c), Burdon-Sanderson (1873) first demonstrated that mechanical stimulation of the trigger hairs is followed by an action potential propagated over the whole surface of the leaf. Thus, continued mechanical

stimulation will cause a continuous production of action potentials (see Chapter 1) and it is proposed that this elicits the release of the low pH fluid observed by Lichtner and Williams (1977) by directly acting on the plasma membrane of the secretory cells.

It should prove possible to test these models by conventional techniques.

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