THE FINE STRUCTURE AND
MECHANISM OF THE LARGE
MECHANOSENSORY HAIR OF
DIONAEA MUSCIPULA ELLIS.

Thesis presented
        by
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        for
the Ph.D. degree of the
University of London.

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PLATE 1.

Three plants of _Dionaea muscipula_ Ellis, showing
developing, open, and closed traps at natural size.

(Colour transparency by courtesy of Professor L.J. Audus).
The diagram below shows the positions of the large,
mechanosensory hairs, usually six in number.
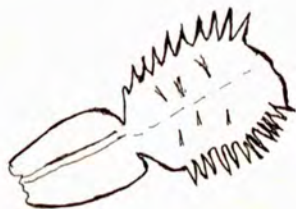
A B S T R A C T

The fine structure of the hinge cells resembled the fine structure of the medullary cells of the hinge region, and of plant or animal tissue capable of active ion transport. There were abundant mitochondria, vesicles and ER. The hinge cell walls possessed numerous plasmodesmata, whose possible functions were compared with those suggested for the Pacinian corpuscle, where degree of stimulus is proportional to the area of membrane distorted.

Closure of traps is a result of bending the sensory hinge, which caused the release of an action potential. Bending the hair caused swelling of organelles, and the appearance of myelin forms in hinge cells, as shown in EM photographs of the hinge region. These effects were considered on the basis of the LUCY model (1964, 1968) for altered membrane phospholipid orientation.

A muscle contracting substance (MCS) was looked for, because acetylcholine has the property of contracting frog rectus muscle, occurs in vesicles in animal synapses as a chemical transmitter of excitation by action potentials, and as well is present in hairs of the plant Urtica. The MCS was identified as LONG'S (1967) 'B' lysophosphatidic acid, resulting from water-soluble phospholipase D acting on lysolecithin.

An action potential is supposed to alter membrane potential and release calcium ions; both would change the activity of phospholipase D. The results following indicated an activated phospholipase from an action potential caused by bending the hair,

caused increased membrane permeability, and trap closure.

Application of chlor-choline-chloride to traps with hairs, suggested activation of lecithin synthesis via activated choline kinase, and reversal of this activation by gibberellic acid.

Current hypotheses, such as an ATP ase "mechanoenzyme" membrane complex altering permeability, the water-filled, phospholipid lined hypothesis of GLAUERT (1968), and the MITCHELL hypothesis (1961), were considered in relation to the results.

A model is presented of <u>Dionaea</u> trap cell with membranes in dynamic equilibrium, a phospholipase destroying, and a choline kinase complex reconstituting the plasma membrane.

ACKNOWLEDGEMENTS.

I wish to express my appreciation to my Supervisor, Professor L.J. Audus of the Department of Botany. He suggested the problem, and guided it. The success of this research is due also to the very broad approach into all aspects of physiology, and to the advice and assistance of many people, a few of whom are mentioned below.

I decided myself to look for a chemical transmitter of excitation by an action potential, and I obtained the resultant conclusion that the action potential caused by touching the large mechanosensory hairs resulted in activation of a phospholipase, which altered the permeability in the traps of <u>Dionaea</u>, and caused them to close.

Advice and Assistance from the Staff of Bedford College.

I wish to acknowledge help given by Professor W.F. Widdas and Mrs. E.L. Hilton of the Department of Physiology in providing facilities for carrying out some of the experiments using frog rectus, and for discussions on aspects of membrane permeability.

Likewise I wish to acknowledge help given by Professor D.F. Cheesman of the Biochemistry Department, for his advice concerning identification of the water soluble, muscle contracting phospholipid.

I was given valuable assistance by the Technician-In-Charge of the Electron Microscopy Unit, Mr. Raynor Jones.

Thanks are also due to Mr. J.D. Valentine, Lecturer in Statistics, who was readily available for consultations regarding biometrical analyses.

Advice from Other Colleges in London.

I received advice from the staff of the Middlesex Medical School, from the Botany Department, Imperial College, and also from Professor C. Long of the Royal College of Surgeons.

Other Research Centres.

Key workers in the field of bioelectrochemistry at Cambridge, co-operated in discussing my results with me. These research workers are mentioned in the literature survey, and include Dr. E.A.C. MacRobbie (University of Cambridge), Dr. Audrey M. Glauert (Strangeways Research Laboratory, Cambridge), and Dr. R.M.C. Dawson (Institute of Animal Physiology, Cambridge.

Finally I wish to thank my former teacher of undergraduate days at Sydney University, Professor R.N. Robertson, now at the Botany Department at the University of Adelaide. He visited London during 1967 for an extended period, and suggested certain lines of approach to this problem.

CONTENTS

CHAPTER 13.

EXPLANATION OF PLATES.

AT	– artifact	NU	– nucleolus
CH	– chlorplast	O	– oil droplet
CU	– cuticle	P	– pore
CW	– cell wall	PB	– protein body
CY	– cytoplasm	PD	– plasmodesma
G	– Golgi apparatus	PL	– plastid
ER	– endoplasmic reticulum	PM	– plasma membrane
H	– hinge indentation	PR	– polyribosomes
M	– mitochondrion	PS	– pore site
ML	– middle lamella	R	– ribosome
MT	– microtubule	T	– tonoplast
N	– nucleus	V	– vacuole
NM	– nuclear membrane	VE	– vesicle

EXPLANATION OF DIAGRAMS SHOWING MAJOR ORGANELLES.
(PLEASE REFER TO KEY DIAGRAMS OPPOSITE).

Endoplasmic reticulum	–	nearly parallel lines
Golgi apparatus	–	as would appear in actual electron micrograph
Mitochondrion	–	solid black
Nucleus	–	hatched
Nucleolus	–	white
Plastids	–	organelles with internal lines.
Vesicles	–	very small circles.

(A). LIGHT MICROSCOPY.

— ML
— CU
— CW
— CY
— V
— N

(B) ELECTRON MICROSCOPY.

— ER
— CU
— CW
— CY
— V
— N
— NU
— M
— G
— G
— PL

VE

ML

CONDUCTING
CELL

HINGE CELL
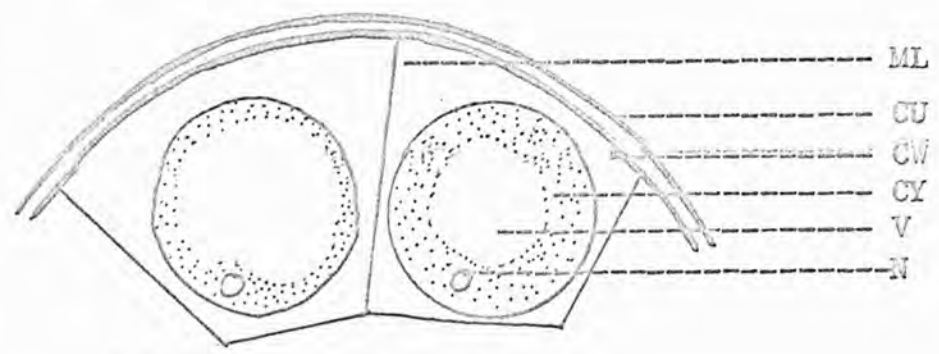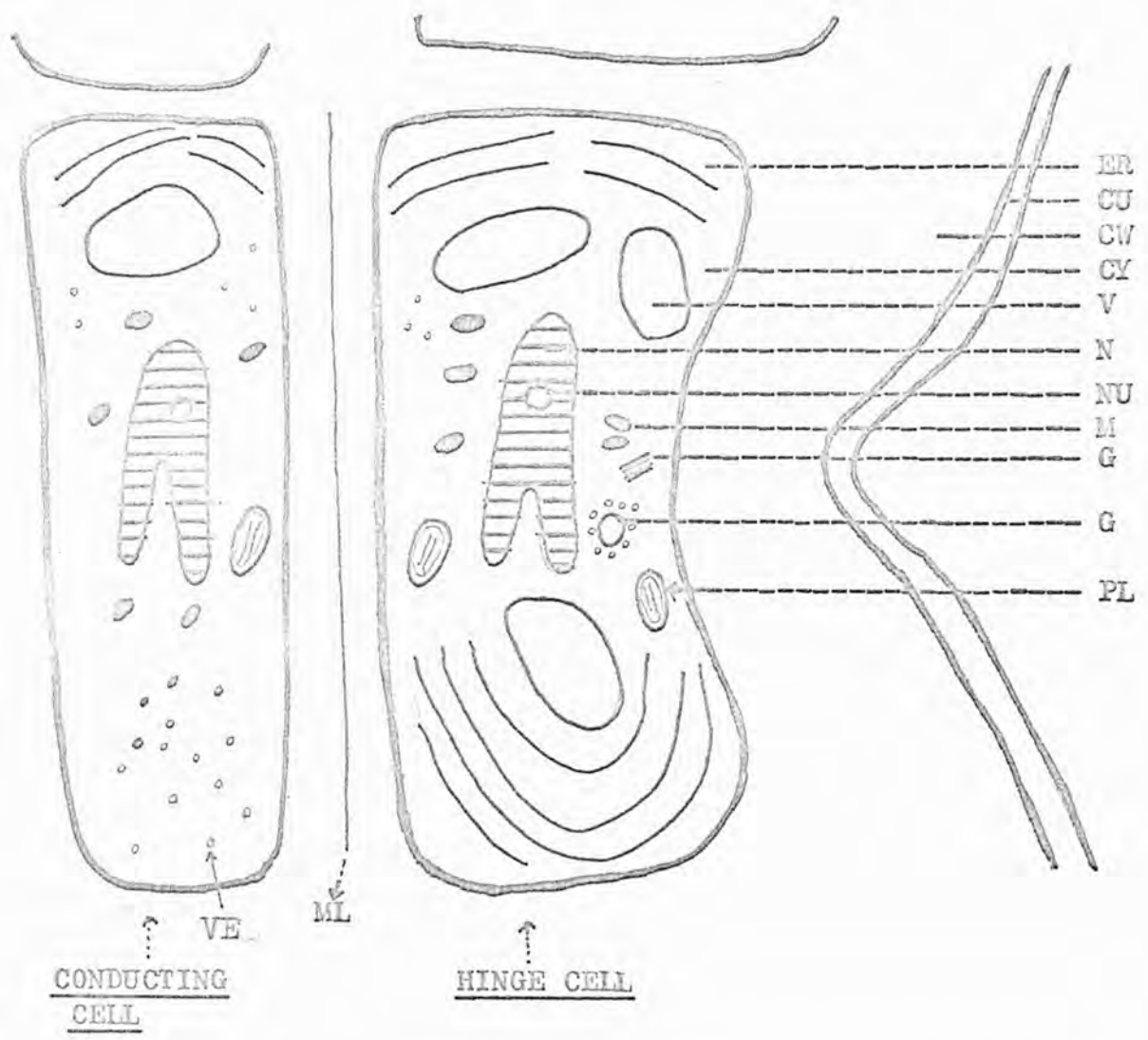
I B KEY DRAWINGS TO ILLUSTRATE DIAGRAMS SHOWN IN PLATES.

1.   GENERAL INTRODUCTION

An essential feature of the large sensory hair of *Dionaea*
is that an action potential is generated in a specialized region
called the "hinge" of the hair with a touch stimulus (DiPALMA,
1966; BENOLKEN & JACOBSON, 1967); this is linked to a sudden loss
of turgor of the inner epidermis of the trap, causing it to
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CHAPTER 1

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INTRODUCTION

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At an early stage in the development of this thesis, a
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and isolation of a substance, later found to be a phospholipid,
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The molecular properties of excitable animal cells have been
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in the receptor, caused by an increase in permeability, and associ-
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in the type of long-term permeability change found in <u>Dionaea</u>

traps caused by stimulating the sensory hair, a phospholipase is activated. Such a suggestion calls for the review of action potentials in plants, bioelectrochemistry, membrane structure and chemistry, phospholipid chemistry, and sense receptor organs generally.

PLATE 2A.

Drawing from light microscope photographs of
a median, longitudinal section of a large,
mechanosensory hair of Dionaea.

X 200

PLATE 2B.

Drawing from light microscope photographs of
a median, longitudinal section of a small
sensory hair.

X 400

2A

LEVER

PODIUM

GLAND

HINGE
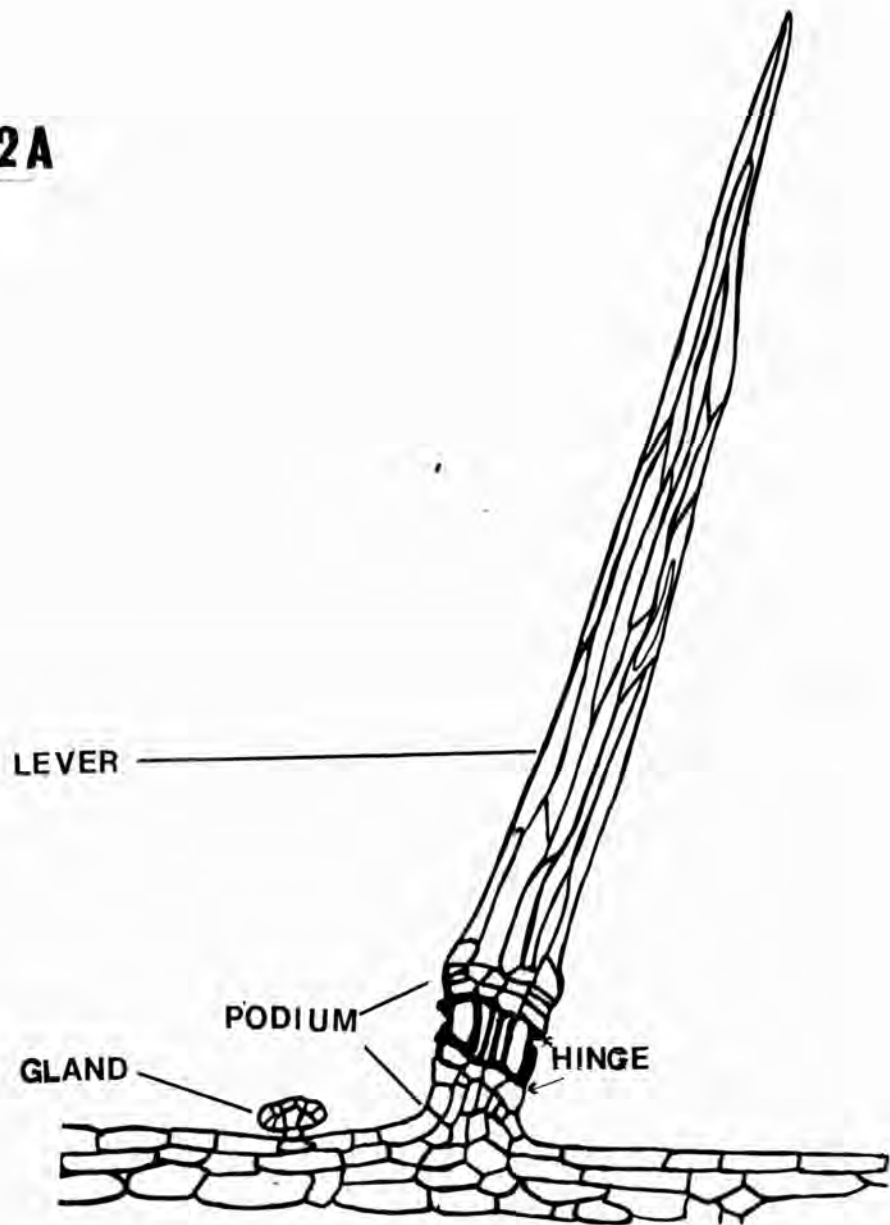
2 B

2. GENERAL DESCRIPTION OF <u>DIONAEA MUSCIPULA</u> ELLIS

The insectivorous plant <u>Dionaea muscipula</u> Ellis or "Venus' fly-trap", was first described by a London merchant John Ellis in 1770 (ELLIS, 1770) in a letter to Sir Charles Linnaeus, as a newly discovered sensitive plant. <u>Dionaea</u> is a native to the eastern part of North Carolina, growing in damp situations, and is a member of the small family, Droseraceae.

The roots of this plant are small, and may consist of only two branches, about 1 inch in length, apringing from a small bulb, as described by DARWIN (1875). The bilobed traps shown in plate 1, page 1B, vary considerably in size within species, but are generally about 1 to 2 ins. long. There are generally 3 large mechanosensory hairs per lobe, triangularly placed.

LLOYD (1942) presented a thorough review of much of the extensive literature concerning the preying sequence, mediated by the sensitive hairs. Each hair is a much differentiated, multi-cellular structure which consists of two morphological regions, the distal level region and the podium region, as shown in plate 2A, page 15. BROWN and SHARP (1910) showed that the podium was the locus of the receptor site. They found that after removal of the lever region, mechanical stimuli delivered directly to the remaining part of the hair (the podium), resulted in closure of the trap. More recently, BENOLKEN & JACOBSON (1967), localised the excitable cells still further, and their date supported the 19th century notion that the sensory organ should be located in

PLATE 3A.

Transverse section through the hinge of the mechanosensory hair of Dionaea. Fixed in Luft's permanganate (note the staining of the cell walls), embedded in araldite, and the section cut ½ micron thick. Phase contrast photograph.

X 1000.

PLATE 3B.

Longitudinal section through the hinge of the mechanosensory hair of Dionaea. Fixation in glutaraldehyde, post-fixation in osmic acid, and the section cut ½ micron thick. Phase contrast photograph.

X 1000.

3A

3B

X 1000.

PLATE 4A.
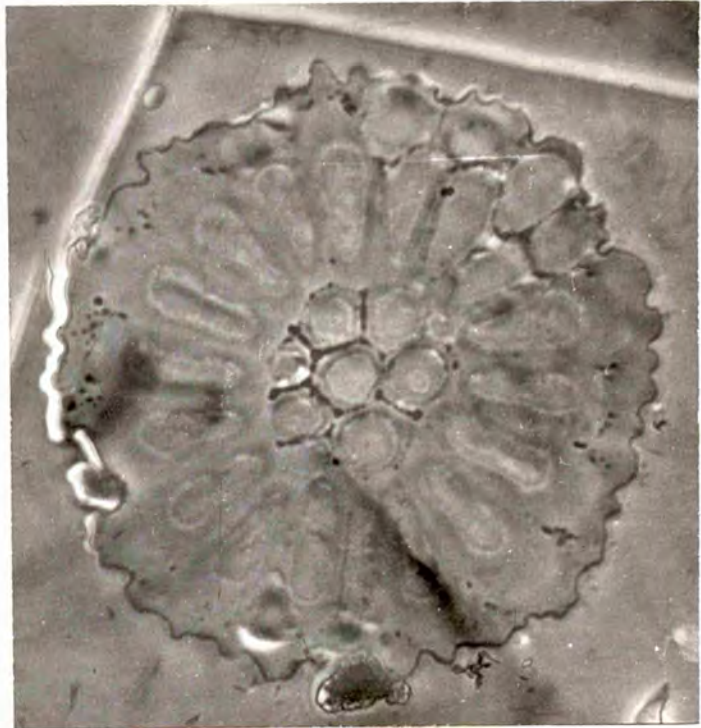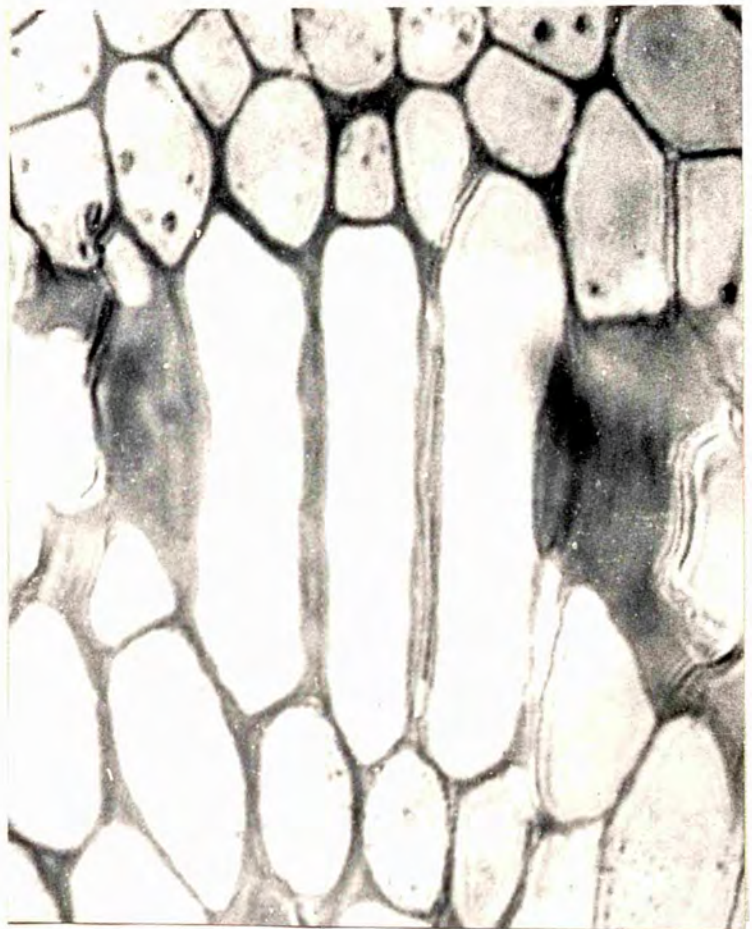
T.S. through large sensory hair at the centre
of the hinge cells. Fixed in glutaraldehyde,
embedded in hard ester wax, and stained for
the light microscope with Sharman's triple
stain, safranin-Orange G and iron-tannic acid.

X 1000.

PLATE 4B.

L.S. through the hinge region of the large
sensory hair of Dionaea. Fixation, embedding
and staining were as above.

X 1000.

18B
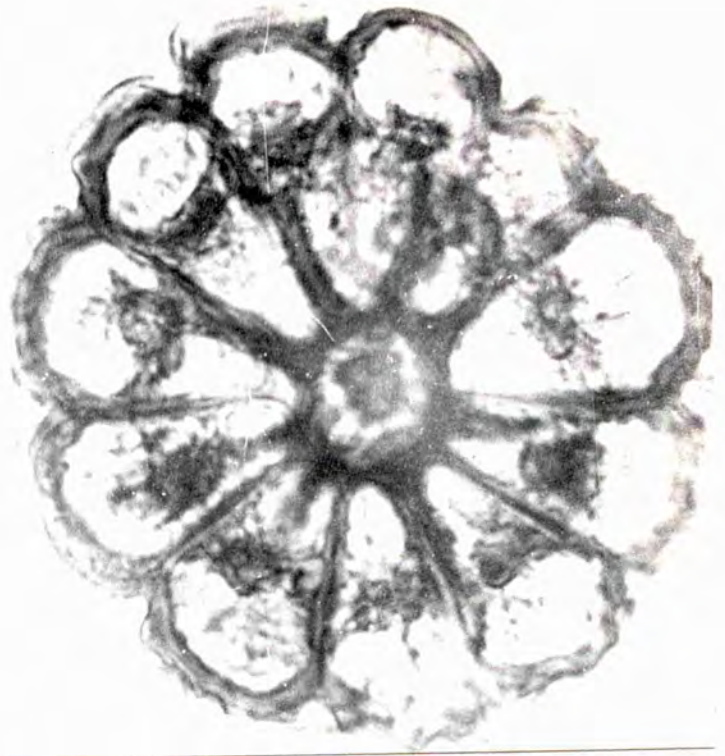
4A

4 B

that layer of cells where there is an indentation (plates 2A and
3B, pages 15 and 17), and the sensory hair bends most easily.
To prove this, the hair was excised and mounted in a chamber for
recording the action potential following mechanical compression.
The hair was sectioned, and the sensory layer found to be that
portion of the podium referred to in this thesis as the "hinge",
and divided into the outer, indented "hinge" cells, and the inner
"conducting" cells, illustrated in plate 15, page15B. The upper
surface of the trap is thickly covered with minute digestive
glands of a reddish colour, as illustrated in plate 9, page 57.
When the large hairs are stimulated, the trap will close, and if
nitrogenous bodies such as meat or gelatine are dampened and enclosed
in the trap, the trap will remain closed (DARWIN, 1875). DARWIN
also described how meat alone could cause the traps to close, and
to secrete an enzyme. Certain proteins alone caused the traps to
close. Another type of gland is for alluring insects. These are
embedded somewhat in the epidermis, whilst the digestive glands
stand out. The alluring glands described by JONES, (1923) are stated
as differing from the digestive glands in lacking the red colour,
and occupy a narrow zone just within the ciliated margin, separated
from the digestive type by a narrow zone free of glands. The
baited marginal band is so situated upon the leaf surface that
a visiting insect too small in length to reach from the bait to
the trigger hairs, usually does not spring the trap, i.e. prey

are normally over 5 mm. long (LLOYD, 1942).

The undersurface of the trap is covered with numerous stellate trichomes, as shown in plate 2B, page 15B. These have been described by LLOYD (1942) and by DARWIN (1875). DI PALMA et al. (1961 and 1966) studied the action potentials which arise by stroking these hairs, and considered that the function of stellate trichomes may be to sensitise the trap, requiring less stimulus when the fly touches the large sensory hairs, to close the trap.

SANDERSON (1874) stated that there is a normal electric current in the trap and leaf blade, and that when the trap is stimulated, the potential difference is disturbed in a similar manner to the way in which a mammalian muscle is excited by a nerve impulse. Since then, these action potentials have been studied by a number of workers, the most recent and most refined study having been made by JACOBSON (1965). The latter continued his work in a paper in which the source of the action potential was shown to be the hinge cells, (BENOLKEN & JACOBSON, 1967).

3. PLAN OF THE WORK

A. Microscopy.

a. Since the published literature on the structure of the large, sensitive hair of <u>Dionaea</u> is limited, especially the fine structure, the first step was to cut sections in both planes, L.S. and T.S. of several hairs, and so get a better idea of the general structure, as seen under the light microscope.

For the study of fine structure, serial sections of several hairs were examined, especially the L.S. of the hinge region, in both the stimulated and unstimulated condition. Factors for examination were movements of organelles, and alteration of fine structure with stimulation; for example, changes in the appearance of membranes.

b. <u>Review of literature for microscopy</u>.

In reviewing the literature, as well as the structure of <u>Dionaea</u>, organs or cells in which an action potential has been studied, or in which active ion exchanges or pumping mechanisms are known to occur, or receptor organs generally, for the whole of biology, have been studied.

Since Vitamin A seems to play such an important part in the stability and permeability of membranes (e.g. DINGLE and LUCY, 1962), then this subject has been looked at also.

B. Mechanism.

As already stated, at an early stage of this investigation,

stimulation of the mechanosensory hair caused the activation of a phospholipase, which led to the formation of a lytic phospholipid. The effects of the action potential on membrane chemistry, the kinetics of its activation, and its location in the sensory hair or trap required determination.

The review of the literature in regard to action potentials and membrane chemistry, and details of the plan of work for this study, have been left until after the discussion of fine structure.

C. Fine structure and mechanism

The relationship between the findings concerning fine structure and mechanism have been discussed, and lines of approach for future research considered.

CHAPTER 2

MICROSCOPY
REVIEW OF LITERATURE

1. LIGHT MICROSCOPE STUDIES

Review of the literature on the structure and mechanism of the larger mechanosensory hair of Dionaea.

A. Structure.

An average large or trigger hair is from 0.75 to 1.5 mm. long, and 0.1 mm. wide at the hinge. It is proportional to the size of the trap.

The lever consists of elongated, thick-walled cells, and any slight movement causes a bending in the basal region, to which OUDEMANS (1859) attributed a special sensitivity. The lever consists of prosenchymatous, living cells which are unlignified (HABERLANDT, 1914).

HABERLANDT divides the podium beneath the lever into three zones:

1. A zone of flattish cells, comprising two or three tiers of central and three or four of epidermal cells. The cells of this zone also invariably contain living protoplasts, in spite of the fact that their walls are more or less suberised.

2. The sensitive hinge of the hair was first described by OUDEMANS (1859). HABERLANDT (1914) described the hinge as being identified by a furrow encircling the hair at this level.

The hinge consists of about ten kidney-shaped cells, plate 4A, B,
page 18, which have a central nucleus, are highly vacuolate and thick
walled, and are modified epidermal cells, surrounding a central core
of tissue, communicating through to this living centre by delicate
protoplasmic fibrils, which actually occur on the radial, longitud-
inal and inner walls of the sensory cells. The hinge is surrounded
by a thick cuticle, which is furnished with numerous minute nodules
and serrations on its inner surface which would serve to anchor it
to the cellulose wall beneath under repeated movements. GOEBEL
(1891) first described the hinge, and noted that under slight stimu-
lus, but sufficient to close the trap, the trigger hair moves at the
hinge, but if the lever region is displaced strongly, then the whole
of the podium moves. GOEBEL noted that there is no vascular con-
nection between the medullary cells of the hinge, and the leaf,
since there is no vein in the hair. HABERLANDT noted a) that the
middle lamellae of the central cells are thick and highly refractive,
that their middle lamellae enclose numerous microscopic granules and
rods of a cutin-like material, and b) that probably the principal
function of the central core was the transmitting of the excitation
produced by deformation of the sensory elements to the pedestal of
the bristle, and thence to the tissues of the lamina.

3. A cylindrical pedestal composed of parenchymatous cells
occurs at the base of the podium. This pedestal is widest above,
and contracts somewhat towards its base. Consequently when the

bristle is violently deflected it bends to a certain extent at the base, so that the delicate hinge is preserved from deformation. HABERLANDT stressed that there is no doubt that the actual perception occurs at the hinge.

The function of the larger mechanosensory hair.

The function of these trigger hairs is to close the traps, and since the time of SANDERSON (1874), this has been understood to be due to electrical stimulation. The threshold stimulus was described by DARWIN as that produced by a piece of human hair one inch long moved once across a single trigger hair. This illustrates the extreme sensitivity of the trigger mechanism.

BROWN and SHARP (1910) related time between stimuli, the number of stimuli necessary and the temperature. At 40^{0}C. only one stimulus was required in 50 per cent of instances. At 15^{0}C. two stimuli were always required. The authors proceeded to determine the number of mechanical stimuli required when at intervals of 1/3, 1, 2 and 3 minutes, and found that 2.0, 3.8, 6.2 and 8.7 stimuli were required, on the average of several tests. They expressed this by the formula:

$$N = 1.2 + 2.5t$$

where N = the number of stimuli and t = the time in minutes between stimuli. This indicated that response follows on a definite amount of accumulated effect, "possibly the accumulation of some chemical substance, as a result of excitation." The total time $N\Delta t$, of a stimulus series could vary from a fraction of a second to at least

5.5 hours for N = 18. That a series of stimuli delivered over
several hours can elicit closure of a trap strongly suggests that a
stimulus "memory" exists in the system.

DENOLKEN (1963) has suggested the following summary of the
known steps in the preying sequence:
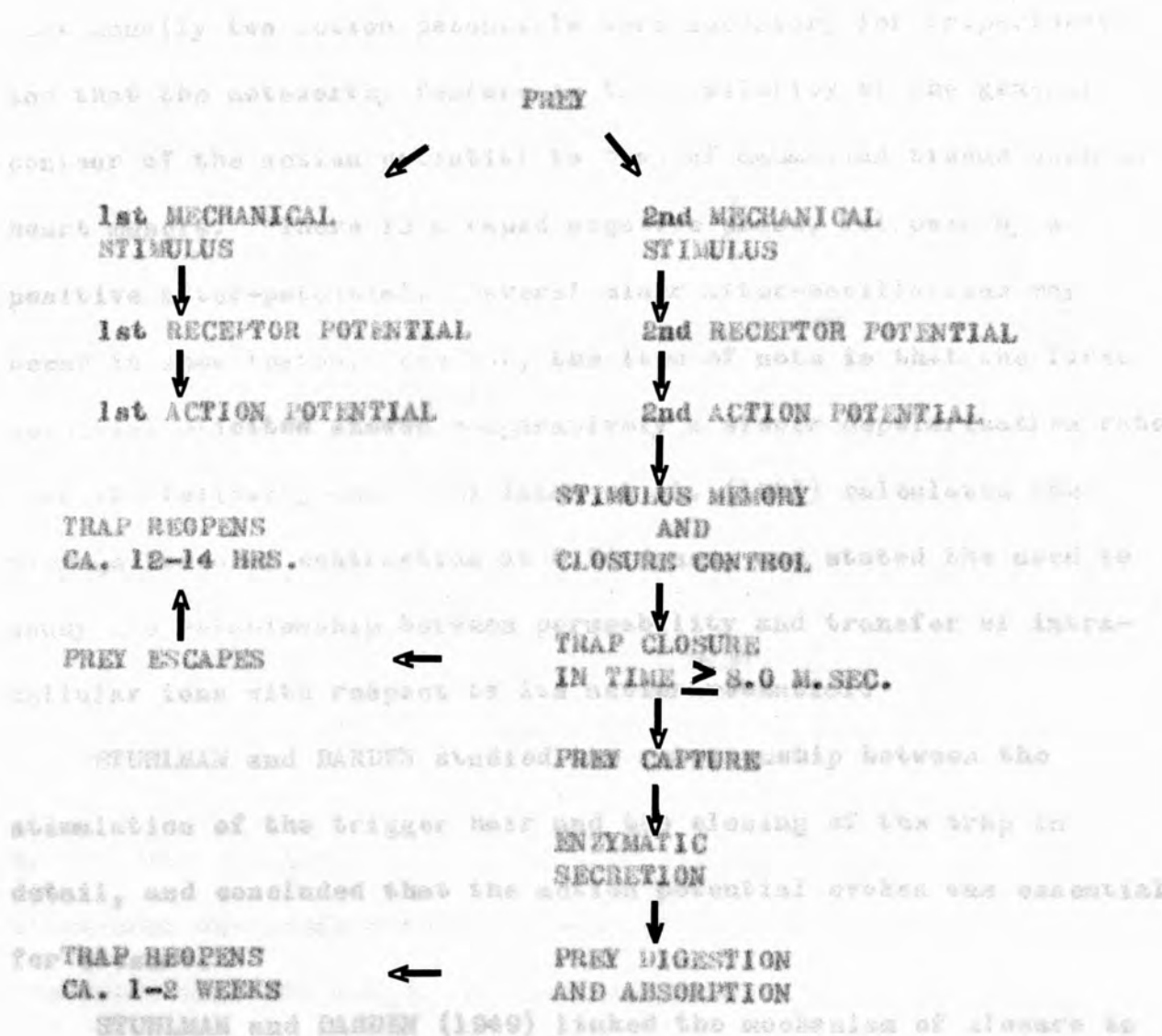
```
                                PREY
                        ↙                ↘
        1st MECHANICAL              2nd MECHANICAL
        STIMULUS                    STIMULUS
             ↓                           ↓
        1st RECEPTOR POTENTIAL      2nd RECEPTOR POTENTIAL
             ↓                           ↓
        1st ACTION POTENTIAL        2nd ACTION POTENTIAL
                                         ↓
                                    STIMULUS MEMORY
                                         AND
    TRAP REOPENS                    CLOSURE CONTROL
    CA. 12-14 HRS.                       ↓
             ↑                      TRAP CLOSURE
    PREY ESCAPES   ←                IN TIME ≥ 8.0 M.SEC.
                                         ↓
                                    PREY CAPTURE
                                         ↓
                                    ENZYMATIC
                                    SECRETION
                                         ↓
    TRAP REOPENS   ←                PREY DIGESTION
    CA. 1-2 WEEKS                   AND ABSORPTION
```

STUHLMAN and DARDEN (1949) linked the mechanism of closure to
a loss in turgor in the leaf trap upon stimulation of the trigger
hair or nearby areas and STUHLMAN (1960) linked closure more
specifically to a sudden reduction in the hydrostatic pressure of

STUHLMAN (1948) found that the more intense the stimulus, the
slower the recovery. STUHLMAN and DARDEN (1950) measured the action
potentials by placing electrodes in contact with the surface of the
trap. DI PALMA et al. (1961) observed that action potentials always
preceded closure of the trap; their experiments at 26°C. showed
that usually two action potentials were necessary for trap-closure,
and that the noteworthy feature is the similarity of the general
contour of the action potential to that of mammalian tissue such as
heart muscle. "There is a rapid negative phase, followed by a
positive after-potential. Several minor after-oscillations may
occur in some leaves. However, the item of note is that the first
potential elicited showed comparatively a slower depolarisation rate
than the following one." DI PALMA et al. (1961) calculated the
average force of contraction at 6.74 dynes, and stated the need to
study the relationship between permeability and transfer of intra-
cellular ions with respect to its action potential.

STUHLMAN and DARDEN studied the relationship between the
stimulation of the trigger hair and the closing of the trap in
detail, and concluded that the action potential evoked was essential
for closure.

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a loss in turgor in the leaf trap upon stimulation of the trigger
hair or nearby areas and STUHLMAN (1950) linked closure more
specifically to a sudden reduction in the hydrostatic pressure of

the cells of the inner epidermis.

The method of measuring action potential used by STUHLMAN (1950) and DI PALMA et al. (1961), was to apply brush electrodes to the outer surface of the leaf blades, and a direct-current amplifier and oscilloscope, with oscillograph used to complete the circuit. This method contrasted with the method used by JACOBSON (1965) in a study of coding of sensory information in Venus' Fly-trap, wherein approximately 0.5 mm. of the tip of one trigger hair was cut off. The cut exposed the medulla of the hair which provided a relatively low impedance current path to the receptor site. A glass pipette filled with 2 m. M.KCl served as an electrode, and was fitted tightly over the sensory lever, and mechanical displacement of the pipette was done at various amplitudes ranging from 10 to 10^3 μ. The reference electrode was a tube filled with 2 m. M.KCl which contacted the surface of the outer epidermis of the trap, approximately in the centre of the triangle defined by the three sensory hairs. Two cotton wick electrodes saturated with 2 m. M.KCl were used to measure propagated action potentials on the surface of the inner epidermis of the trap relative to the reference electrode. One of the wick electrodes was located near the base of the cut sensory hair, and the other near the margin of the trap lobe.

JACOBSON (1965) recorded non-propagated potentials and found that these non-propagated potentials always precede the propagated action potentials. The receptor potential appeared to couple the mechanical stimulation step to the action potential step of the

preying sequence. JACOBSON presented evidence which supported the
hypothesis that the positive and negative receptor potentials which
he measured, originated from independent sources. JACOBSON analysed
a) the relation of the parameters of mechanical stimuli to the mag-
nitude of the receptor potential, and b) the relation of the receptor
potentials to the action potential and found that the hypothesis
that the positive receptor potential is the generator of the action
potential is consistent with the data.

JACOBSON noted that two characteristics of the _Dionaea_ stimulus-
response relation recall the stimulus-duration curve of nerve axon
(see KATZ, 1939). "In _Dionaea_ shorter duration positive receptor
potentials are elicited by faster-rise stimuli for which the action
potential threshold increases. Hence action potential threshold
increases as the duration of the positive phase of the receptor
potential decreases. Similarly in nerve the threshold for elec-
trical stimulation increases as stimulus duration decreases.
Furthermore, in _Dionaea_ action potentials are not elicited for the
very short duration positive transient of the negative receptor
potential. Similarly, in nerve, action potentials are not elicited
by electrical stimuli of very short duration, regardless of
stimulus amplitude."

2. ELECTRON MICROSCOPE STUDIES

SCALA et al. (1968) have compared three different kinds of plastids of the Venus's-fly trap, which occur as the chloroplast of the leaf cells, the plastids of the cells of the digestive gland, and the plastids of the trigger (hinge) cells. They have noted differences between these three types of plastid, and have attempted to attribute some special function to them in the hinge cells, possibly as capacitators.

3.   FINE STRUCTURE

The sensory hair of Dionaea in relation to the neuron, the synapse
and animal receptor organs.

A.   The neuron.

The neuron contains a nucleus, mitochondria, microtubules and
neurofilaments, as reviewed by PETERS (1968). In addition, there is
the Nissl substance (equivalent to rough ER) described by light
microscopists as having an affinity for basic dyes, and appearing
in a granular form, or as small blocks. DUNCAN (1967) reviewed the
Nissl substance because of theories associating it with memory;
memory has been associated with RNA, and with polypeptides.

Under the electron microscope, the Nissl substance has been
identified as being the granular endoplasmic reticulum with its
surface ribosomes, and free ribosomal particles. The Nissl substance
must be considered in relation to the sensory hair of Dionaea,
because of the "memory" of subthreshold stimuli possessed by Dionaea,
and described by JACOBSON (1965).

B.   The nerve-muscle synapse.

All synapses contain numerous vesicles 150Å to 700Å in dia-
meter, for a wide range of tissues and species; thus for mammals the
diameter is within the range 200Å to 500Å, and for the neuroelec-
troplaque of certain electric fish, 150Å to 250Å. BULLOCK and
HORRIDGE (1965) stated that the occurrence of vesicles is commonly
used as an indication of the presence of a synapse; further, that

evidence of this characterisation is of four kinds:- a) they occur consistently, concentrated at synapses and scattered or rare elsewhere in the neuron; b) their number is said to change when the nerve is stimulated; c) their presence suggests a basis for quantitative release of transmitter substance in packets, and d) vesicles can be observed to open into the synaptic cleft as if releasing their contents.

The occurrence of relatively large quantities of acetylcholine in the hair of Urtica (EMMELIN and FELDBERG, 1947) and the fact that acetylcholine is a common chemical transmitter in the nerve-muscle synapse, focuses attention on these vesicles. Vesicles occur also in certain animal receptors.

Another feature of synapses described by DE ROBERTIS (1964), is the concentration of mitochondria and neurotubules.

C. Other animal receptor organs.

a. Stretch receptors in animals.

The frog muscle spindle has been described in great detail by KARLSSON et al. (1966). The sensory endings are stimulated by contraction or stretching of the intrafusal muscle fibres. These sensory endings are shown to be packed with mitochondria, and to have some vesicles, about 800 $\overset{o}{A}$ in diameter.

b. The Pacinian corpuscle.

The fine structure of the Pacinian corpuscle has been described by PEASE and QUILLIAM (1957). The functional portion is

known to be the central axon, and this is packed around the edges with numerous mitochondria, and contains numerous small vesicles.

c. Fine structure of the cochlear hair cells of the ear.

ENGSTROM & SJOSTRAND (1954) described the large hair cells of the guinea pig cochlea as having about 14 parallel layers of ER parallel to the plasma-lemma, and numerous mitochondria only about 0.15 μ in diameter near the outer membranes. The mitochondria on the periphery indicate the site of energy transformation, according to the above authors. SMITH & DEMPSEY (1957) found a similar picture, but also described numerous small vesicles, and groups of parallel cisternae near the nucleus.

d. Fine structure of the taste bud.

FARBMAN (1965) has contributed a detailed study of the fine structure of the taste bud of rats, and discussed his results in relation to other such studies. The type 2 taste receptor cell has very large numbers of mitochondria, and several types of vesicles and vacuoles, suggesting that it is metabolically the most active cell in the taste bud, and that it is involved in exchanges of large amounts of material between the cell and its environment. There is a Golgi apparatus. The vesicles are thought to represent a specific interaction with the surrounding epithelial cells, although these vesicles could represent part of the Golgi apparatus, or be homologous with synaptic vesicles, but this is considered unlikely.

4. PLANT CELLS AND ORGANS

A. <u>The fine structure of Nitella (relationship to action potentials)</u>.

The fine structure of <u>Nitella</u> is relevant to the study of

<u>Dionaea,</u> because it has been the object for the study of action

potentials by MACROBBIE (1965 and 1966).

NAGAI and REBHUN (1966) made a study of the fine structure of

<u>Nitella,</u> and found an association between bundles, composed of 50 -

100 microfilaments, each 50Å in diameter, and many microns long,

oriented with their long axis parallel to the direction of stream-

ing. The types of fixation used were also relevant to a study of

fine structure in plant cells known to give rise to measured action

potentials.

B. <u>The fine structure of phloem</u>.

Certain literature concerning the fine structure of phloem is

reviewed, because of the appearance of stacked membranes of endoplas-

mic reticulum observed by ESAU and CRONSHAW (1968) and NORTHCOTE

and WOODING (1966). Since frequently the hinge cells of <u>Dionaea</u>

sensory hairs also contain stacks of ER, then the fine structure of

phloem requires attention.

ESAU and CRONSHAW (1968) found with <u>Cucurbita</u> sieve elements,

that there was no apparent association between ER with plastids,

although NORTHCOTE and WOODING (1966) had interpreted their findings

with <u>Acer</u> phloem to indicate an involvement of the ER in plastid

development. NORTHCOTE and WOODING (1966) also visualized a

connection of the ER with the development of protein bodies, but
ESAU and CRONSHAW found no consistent association between the two.

C. Other organs of active ion exchange. Raphide-forming cells.

The significance of this review is that the appearance of the
fine structure of the hinge cells of the sensory hair of Dionaea,
and of raphide-forming cells of Vanilla and Monstera when fixed with
$KMnO_4$, is very similar.

MOLLENHAUER and LARSON (1966), in reviewing raphide-forming cells,
stated that the cytoplasm of cells destined to produce raphide crys-
tals can be differentiated very early in development; clear coinci-
dent patterns of change in plastids, ER, vacuoles and crystal com-
plexes indicate an interrelationship among these subcellular
componants in the production of raphide crystals. These workers
described the plastids as a previously unknown plastid form, with a
general morphology characteristic of proplastids and without
accumulated starch. Mitochondria were abundant, there was a high
organelle to cytoplasm volume ratio, and copious quantities of
tubular ER, often in large numbers of parallel lines, appearing to
be engaged in secretory activity, the product being mobilized in
vesicles and deposited in the central region of the cell. The plastid
alteration during crystal formation is particularly striking, and
was observed in various Cattleya orchids, and other raphide cells.
Portion of the plastid is often modified to form a pyrenoid-like
modification, which have been implicated in the formation of lipid

in cells that do not accumulate starch.

D. The digestive gland of Dionaea.

SCALA et al. (1968) have described the fine structure of the digestive gland of Dionaea. They found that the stalk and basal cells contained numerous lipid globules, and that the common wall between the two cells is traversed by plasmodesmata. The gland is well adapted to its function of protein digestion by having much granular ER and numerous Golgi bodies, ribosomes and mitochondria. Protein in the vacuoles of the resting gland probably consists of zymogens and therefore provides digestive enzymes.

E. Vitamin A studies, fine structure, and permeability.

The functioning of the hair of Dionaea is not necessarily related directly to Vitamin A studies, but certain electron micro-graphs produced by DANIEL, DINGLE et al. (1966) of tissue treated with excess Vitamin A will be shown to have a membrane structure similar to that of the hinge of the Dionaea hair after stimulation — distension of the membranes, swelling of organelles, and the formation of myelin-like structures.

LUCY and DINGLE (1962, 1964) suggested that Vitamin A may act in the stabilisation of membranes by acting as a cross-linking between lipids and proteins. The projecting methyl groups of Vitamin A may bind to those of cholesterol by Van der Waal's forces, whilst its hydroxyl group may be attached to a protein molecule.

LUCY and DINGLE (1962) found that Vitamin A controlled

permeability up to 6 µg/ml at 37° in erythrocytes, and then haemo-
lysis occurred. Lysolecithin also affected permeability and caused
haemolysis at higher concentrations, but its effects were considered
to be different from those of Vitamin A, because of different tem-
perature relationships and because of results from various animals.
LUCY and DINGLE (1962) suggested three possible methods of action
of Vitamin A in altering permeability; a) the compound may activate
enzyme systems which can attack the membrane; b) Vitamin A may
become attached to the structural components of membranes, so that
the normal molecular components of the membrane are displaced;
c) Vitamin A may interfere with reactions essential to the maintenance
of the membrane.

DUNCAN (1967), in reviewing transducer mechanisms, described how
Vitamin A is essential for taste and smell, and combined with
Rhodopsin is needed for sight. The evidence suggested the activity
of a contractile protein in the membrane, activated by an ATP –
ATP ase system via Vitamin A, in each case.

METHOD OF SURVEY

... re and fine structure of antennae they had.

... ... details of fine structure ... ...

... ... because of the ... ...

... serial sections were cut ... with ... ...

... the longitudinal plane, and ... ...

... in the transverse plane, for ... the sections ... ...

light microscope. Particular attention ... paid to ... ...

the hinge region.

C H A P T E R   3

Electron Microscopy.

... ... hairs were studied in the

... ... state. Serial sections were cut, and detailed studies

M E T H O D   O F   S U R V E Y

... ... section longitudinal section of the hinge. Drawings were

M I C R O S C O P Y

... ... into sections (these sections were not used for drawing,

because these sections were orientated slightly obliquely), and both

glutaraldehyde-osmic acid fixed, and permanganate fixed material

examined.

Another five sections were examined in the stimulated position,

and this was done by bending the hair, and fixing it in the bent

position.

Observations were made on the appearance of fine structure

before and after stimulation, especially in changes in the hinge

cells, their cell walls, and their contents, as well as in the

movement of organelles.

METHOD OF SURVEY

Cell structure and fine structure of mechanosensory hair.

Before the details of fine structure of the sensory hair were explored, and because of the lack of detailed published work on its structure, serial sections were cut of three hairs from different traps in the longitudinal plane, and of three hairs from different traps in the transverse plane, for a detailed examination under the light microscope. Particular attention was paid to the MLS, and to the hinge region.

Electron Microscopy.

Sections from eight different hairs were studied in the unstimulated state. Serial sections were cut, and detailed studies made of the median longitudinal section of the hinge. Drawings were made from five sections (three specimens were not used for drawing, because these sections were orientated slightly obliquely), and both glutaraldehyde-osmic acid fixed, and permanganate fixed material examined.

Another five sections were examined in the stimulated position, and this was done by bending the hair, and fixing it in the bent position.

Observations were made on the appearance of fine structure before and after stimulation, especially in changes in the hinge cells, their cell walls, and their contents, as well as in the movement of organelles.

CHAPTER 4

METHODS IN MICROSCOPY

PRELIMINARY LIGHT MICROSCOPE STUDY METHODS

LIGHT MICROSCOPY - WAX EMBEDDING

1. Fixation. Two fixatives were used:-

A. Farmer's fluid (acetic-alcohol).

Fixation time was 20 minutes at room temperature, followed
by 5 changes of 95 per cent alcohol, and dehydration in absolute
alcohol.

B. Glutaraldehyde in phosphate buffer.

2 per cent glutaraldehyde in M/10 phosphate buffer at pH 7.4
and 0°C. for 12 hours, plus aspiration at a vacuum equivalent to
29 inches of mercury for 15 minutes. This gave better results.

2. Dehydration and embedding.

Three methods were used:-

A. A graded water-ethanol series, then embedded in paraplast
using either a xylol or chloroform method.

B. A graded water-ethanol-tertiary butanol series, ending in
100 per cent TBA, and then into paraplast wax (JENSON, 1962).

C. The best method was that used by CHAYEN and GAHAN (1959),
and consisted of dehydration in a graded water-acetone mixture to
absolute acetone (see Appendix), and transfer to "Ester Wax 1960"
as used by BAKER and JORDAN (1953) and STEEDMAN (1957).

3. Cutting.

With ordinary wax, the sensitive hair of Dionaea was difficult
to embed, and was found to be brittle.

Using hard ester wax, and the rapid method of embedding of

CHAYEN et al. (1959), the hair could be sectioned serially, easily. Sections in the longitudinal plane were cut at 4 μ thickness to see detail, and in the horizontal plane it was only necessary to cut at 8 μ thickness.

Expansion of sections in ester wax was best done on distilled water at room temperature, without applied heat.

4. Staining.

A satisfactory stain was the triple stain of tannic acid-iron alum with saffranin and orange G, used by SHARMAN (1943).

5. Orientation for wax embedding.

Orientation for sectioning in the longitudinal plane, alone presented difficulties, because of the small width of the hair, 50 to 150 microns diameter.

The method of orientating on a stained pith surface described by SHARMAN (1960) was used. Orientation was achieved using a 4 per cent aqueous solution of sodium alginate, which is caused to be converted into a gel by a calcium chloride solution, and then fixed. The disadvantage of this method is that the alginate appeared in photographs of sections on the outside of the specimen.

It was found that most hairs of Dionaea are accurately aligned at right angles to the mid-rib, and it was only necessary to section at right angles to the mid-rib to get good longitudinal sections of the large mechanosensory hair.

6. Plastic embedding.

As a routine process of trimming blocks for electron microscopy, sections from 1,000 $\overset{o}{A}$ to 5,000 $\overset{o}{A}$ in thickness were cut using a glass knife, and transferred to a glass slide. The section was allowed to dry down to the slide and then examined, either by phase-contrast or by staining.

7. <u>Histochemical test for cholinesterase.</u>

For this test, hand cut sections were used.

A modification of Koelle's method was used (see Appendix, Table 2, page 275). The cholinesterase, if present in hand sections of the hinge of the sensitive hair, hydrolyses acetylthiocholine added as reagent. Copper sulphate also added causes copper-thiocholine to be precipitated at the site of the enzyme. On treatment with ammonium sulphide the precipitate is transformed to black copper sulphide.

The significance of cholinesterase is that it is present in the animal nerve-muscle synapse to break down acetyl choline after the latter has allowed a nerve action potential to pass. The sensory hair hinge of <u>Dionaea</u> has certain features in common with the synapse, and therefore it is of interest to see if acetylcholinesterase is present in the hair of <u>Dionaea</u>.

METHODS — ELECTRON MICROSCOPY

1. Fixation.

Two types of fixative were used:-

A. Luft's permanganate.

A satisfactory method was to fix the whole trap at $0^{o}C$ for 30 minutes, whilst aspirating, to wash in distilled water, dissect out the sensitive hairs, and fix again for another 5 minutes at $0^{o}C$, then to wash and dehydrate for embedding.

Fixation in Luft's permanganate was found to be a useful adjunct to the use of glutaraldehyde, but tissue preservation was not as good.

B. Glutaraldehyde followed by osmic acid.

Various formulae were tried, and the following gave the best results:-

a. 2 per cent glutaraldehyde in M/10 phosphate buffer, pH 7.0, $0^{o}C$, fixation time 10 hours, aspirated.

b. Washed in M/10 phosphate buffer, pH 7.0, $0^{o}C$ for 4 hours.

c. 2 per cent osmic acid in M/10 phosphate buffer, pH 7.0, $0^{o}C$, fixation time 3 hours.

d. Washed in M/10 buffer at $0^{o}C$ for 3 hours, then dehydrated.

The above method is based on the recommendations of GUNNING (1965).

Sucrose and manitol were both used to control osmotic pressure, but equally good results were obtained without sugar.

KAY (1965) stressed the need to use pure glutaraldehyde. The
pH of 25 per cent glutaraldehyde used as stock falls below 3.5 due
to oxidation, and for good fixation the solution can be purified by
adding a small quantity of barium carbonate, and centrifuging.

2. Dehydration.

Dehydration was through a graded series of ethyl alcohol -
25, 50, 75, 85, 90 and 95 per cent, 5 minutes in each solution, and
final transfer to absolute alcohol (two changes, 1 hour in all).
The tissue was then transferred to propylene oxide (two changes,
1 hour in all.

3. Embedding.

The tissue was then transferred in the following stages to pure
epoxy resin:-

   i) Propylene oxide   resin, 2:1 for 2 hours.
   ii) Propylene oxide   resin, 1:1 for 1 hour.

   After this period it was found to be essential to dissect
away the "lever," and most of the podium below the "hinge" cells,
for good embedding.

   iii) Specimens were transferred to fresh propylene oxide   resin,
1:1 and left overnight in a large dessicator.

   iv) The specimens were transferred to pure resin, which was
changed twice daily for 3 days, with evacuation to remove air and
propylene oxide during the first change.

   LUFT (1961) recorded that residual traces of propylene oxide

can enter into chemical combination with the polymerising resin,
whereas alcohol cannot.

For orientation, it was found that usually perfect alignment
for L.S. could be obtained by polymerising in a small, single drop of
resin on a sheet of polythene 1/16 inch thick. After polymerisation
for 24 hours at 60 $^{\circ}$C, the drop was transferred flat side down onto
the thin, flat end of a resin blank moulded in a BEEM polythene
capsule, which had been smeared with a little araldite adhesive.
Further polymerisation for 24 hours at 60°C was given.

4. Resin mixtures.

i) Epon.

Various mixtures were used, including variations of those
used by LUFT (1961).

The most suitable mixture was that set out on the following
page , and used by Dr. R. Coleman, Bedford College, London, N.W.1.
Small quantities of nearly 8 mls. could be made up at a time, mixed
in a mechanical tumbler for one hour, and air bubbles removed in a
vacuum for 3 minutes. The mixture could be stored for some weeks at
-15°C.

Preparation of epon mixture (COLEMAN)

Ingredient	Specific gravity	Volumes for large quantities (mls.)	Mass of ingredients for small quantities
Epikote 812	1.2	50 ml.	3.00 g.
D.D.S.A. (dodecenyl succinic anhydride)	0.98	80 ml.	3.90 g.
M.N.A. (methyl nadic anhydride)	1.19	10 ml.	0.65 g.
B.D.M.A. (benzyldimethylamine)		2.7 ml.	0.135 g.

Glassware is cleaned afterwards in 1) acetone,

2) detergent.

Araldite

Good results were obtained with Araldite, provided that a diamond knife could be used. The mixture used was as follows (KAY, 1965):-

	By volume (ml.)	By weight (grams)	Relative density (gram per c.c.)
Araldite M. (or CY 212)	10.0	11.3	1.135
D.D.S.A.	10.0	10.0	0.999
D.M.P. 30 accelerator[x]	0.5	0.5	0.970
Dibutylphthalate	1.0	1.0	1.038
M.N.A.	--	--	1.237

[x]2,4,6 tri (dimethyl amino-methyl) phenol.

It was found convenient to mix the relatively small amounts by dispensing gravimetrically and tumbling.

5. Sectioning blocks.

Trimming was done under a binocular microscope, using a new stainless steel razor blade. The best facet was a truncated pyramid, about 200 μ at the broadest width and only 200 μ long.

Sections were cut on a Huxley microtome with a motor drive. Glass knives were made using an LKB knife-maker, and sections were floated onto distilled water.

Araldite sections were expanded using xylene or chloroform, and epon 812 sections were expanded with dichlorobenzene.

Copper "New 100" grids were used. They were coated with a very thin layer of formvar, and the underside was coated with a thin layer of evaporated carbon. The sections were picked up on the formvar side, usually from underneath. "Silver" and "Gold" sections were used normally.

6. Staining.

Various staining schedules were tried, but the best results were found from the following:-

Staining was done in small tubes, and the grid never allowed to contact a liquid surface during the staining operation. Decarbonated distilled water was used.

A 1 per cent solution of aqueous uranyl acetate was freshly prepared, centrifuged, and the grid stained in the supernatant for

15-20 minutes at 20°C. The grid was washed four times in distilled water, and placed for 5 minutes in the freshly centrifuged super-natant of Reynold's lead citrate. After thorough washing in at least 5 changes of distilled water, the grid was carefully dried.

7. Examination in microscope.

The sections were examined in an A.E.I. EM6 B microscope. Normally sections were examined at 60 K.V., but where sections showed too low contrast 40 K.V. was sometimes used, and for somewhat thick sections 80 K.V. was used.

Photographs were taken on Ilford N 50 plates and processed in Ilford ID 36 P.Q. developer, fixed with Kodafix.

8. Method of fixing the hair in the bent position.

A saturated solution of gelatin in distilled water at a temper-ature of 40°C. was made. The tip of a pair of fine forceps was dipped into the gelatin solution at 40°C., and under a binocular microscope the tip of the mechanosensory hair was bent to touch the epidermis of the trap, whilst 2 per cent glutaraldehyde in M/10 phosphate buffer at 0°C. was dropped on. The gelatin solution became hard immediately, and the whole trap was fixed in the normal way. After fixation of the whole trap in this way, a small block of tissue consisting of the bent hair and the adhering epidermis was post-fixed in osmic acid. After dehydration, and then passage through propylene oxide, the actual hinge region could be dissected away, and remained bent.

The time between stimulus, bending, and the commencement of fixation was less than 2 minutes.

CHAPTER 5

RESULTS

LIGHT MICROSCOPY.

RESULTS

Light Microscopy

In considering these results reference should be made to Plate 2, page 15. The term "podium" will be used to refer to the pedestal on which the "hinge" rests; above the podium is a special layer of cells called the "hinge" which is responsible for the special sensitivity of the hair. The diameter of the hinge varies according to the size of the trap, and ranges from 50 μ to 160 μ. The central core of the hinge will be called the "conducting" cells, because HABERLANDT (1914) supported the view of early workers, that these cells probably function in the transmission of the sensory elements to the podium of the hair, and thence to the trap. As seen in photographs 3A and 4A (pages 17 and 18), the number of conducting cells varies from 1 to 6 (or more), although it is commonly 3 or 4. Around this central core there are about 12 kidney shaped cells, named here "hinge" cells, and these are commonly 80 μ long. The serial sections shown in the drawings in Plates 5, 6, 7 and 8 (pages 54 and 55) show the distinctive features of the hinge, compared with the tissues above and below.

Above the "hinge," there are two or three layers of flattish cells, which have rather thick walls and no intercellular spaces. above these cells rests the "lever," composed of elongated, thick-walled cells, found to be not sensitive by various workers.

Although the transverse section shown in Plate 4A, page 18

might indicate that the nuclei of all the cells in the hinge are in
the one plane, this is not so, as shown for example in 4B, page
18.

The transverse section, fixed in permanganate, embedded in
araldite, and shown in Plate 3A, page 17 indicates deeply staining
tissue in the middle lamellae of the conducting cells, and pore-
like regions in the outer circumference of the hinge cells.

Drawings from sections of the digestive gland are included in
Plate 9, page 57, for purposes of comparison with the sensitive
hair.

PLATES 5, 6 and 7.

Drawings of serial sections through the podium and
hinge regions of the large, mechanosensory hair.
These transverse sections were prepared from a
hair fixed in glutaraldehyde, embedded in hard
ester wax, and stained with Sharman's triple stain.
Sections 8 to 14 are through the "hinge" cells.

X 1000.

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<u>PLATE 8.</u>
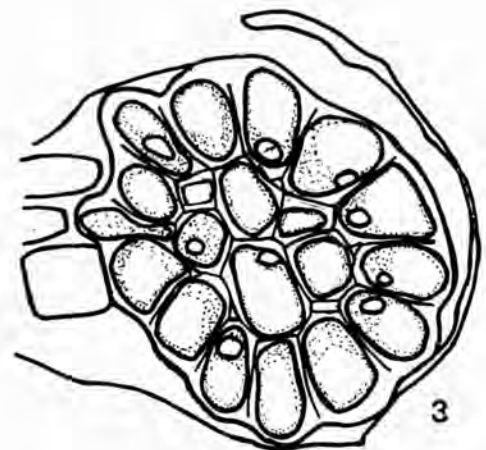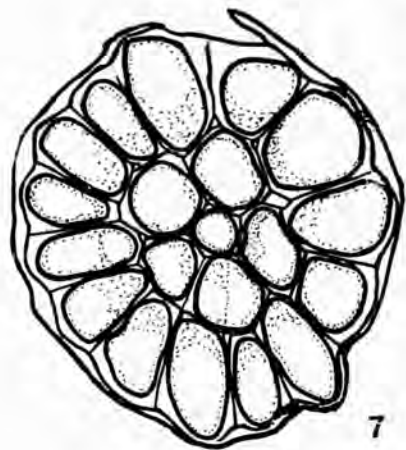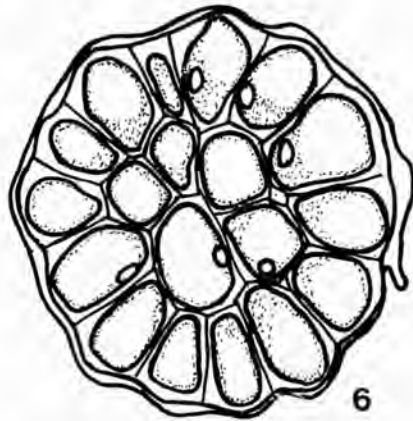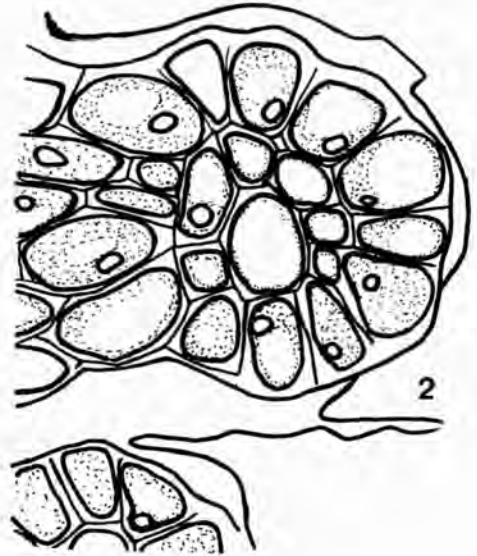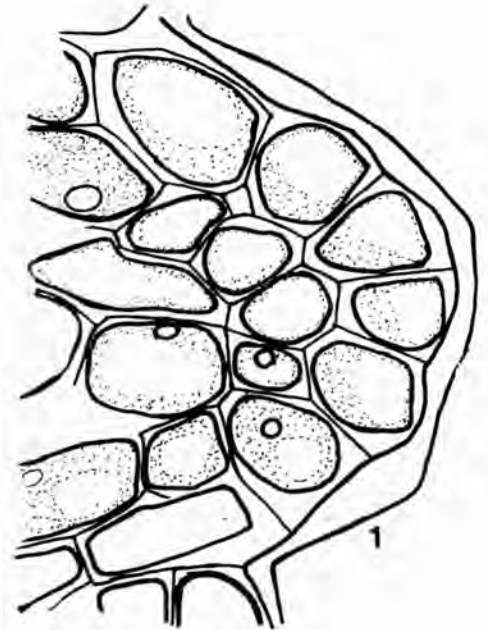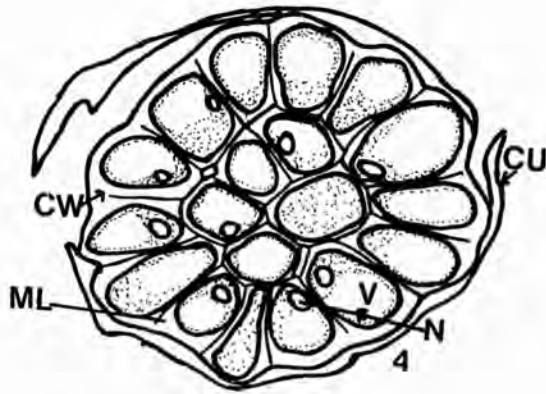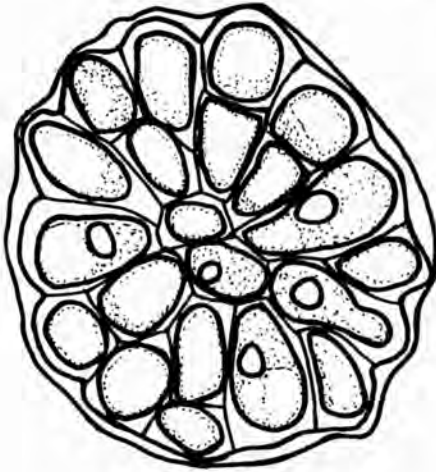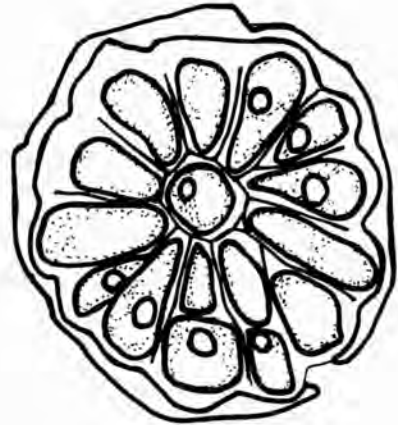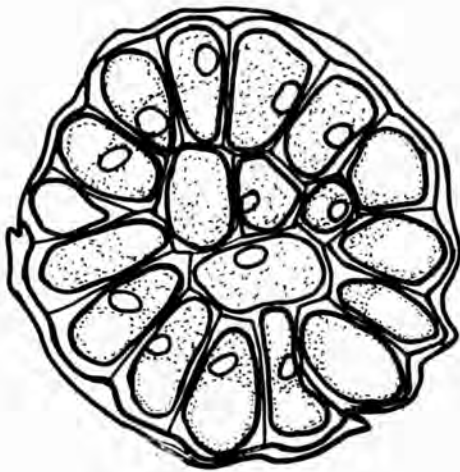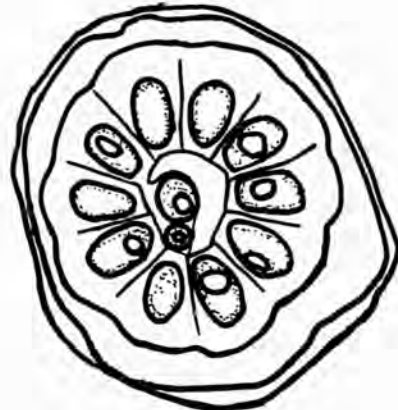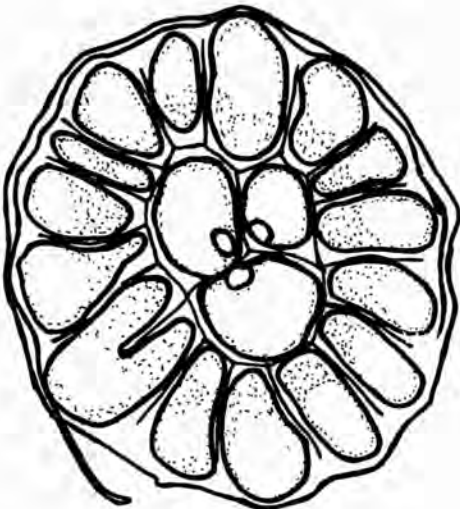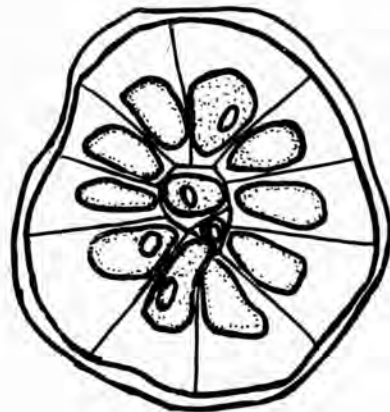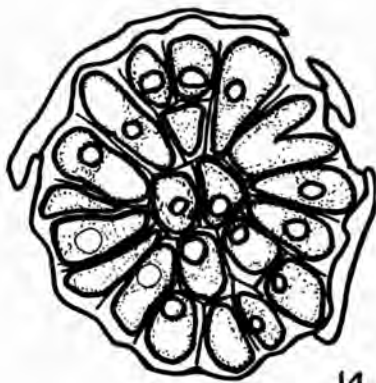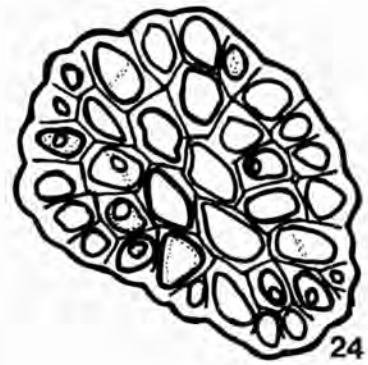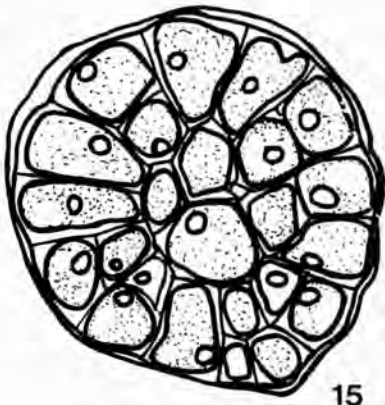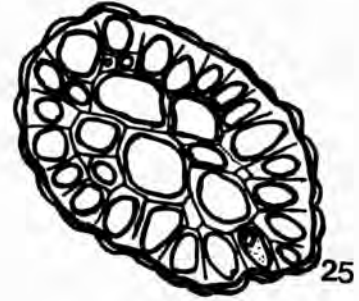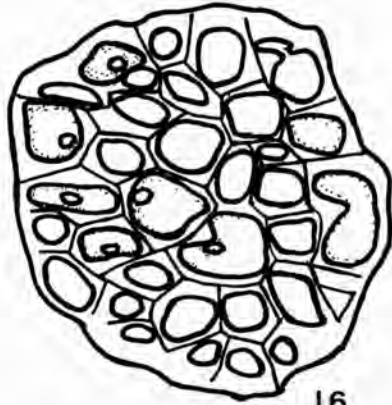
Drawings made from photographs of longitudinal
sections of the base of the large, sensory hair
of <u>Dionaea</u>. "D" is a section in the median
position. The drawings are from serial sections,
cut at a thickness of 5 microns and representative
of four positions in the hair. Fixed in
glutaraldehyde, embedded in hard ester wax, and
stained in Sharman's triple stain.

X 1000.
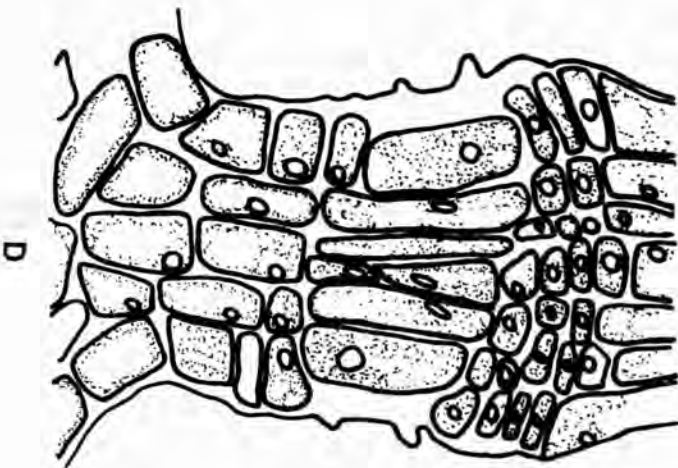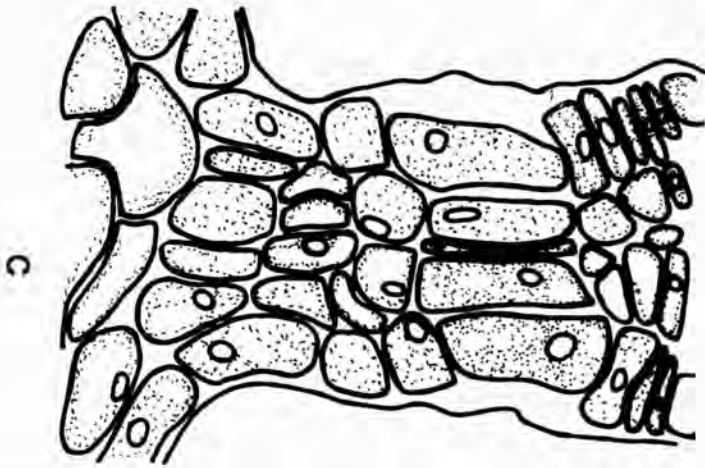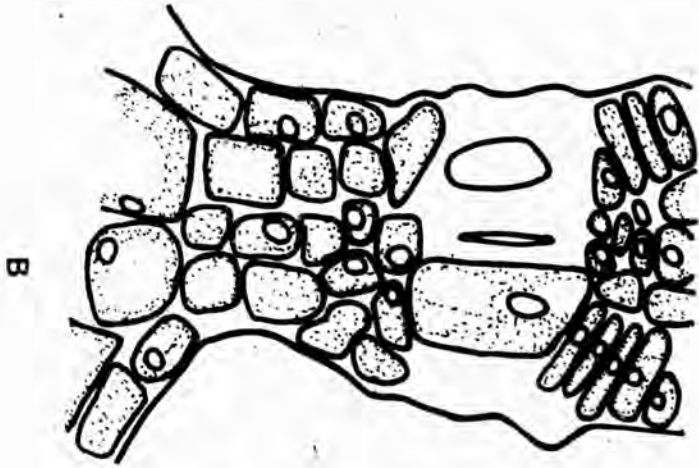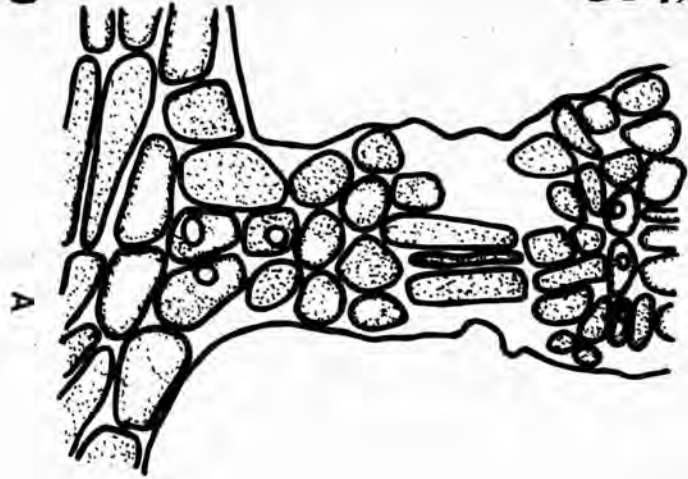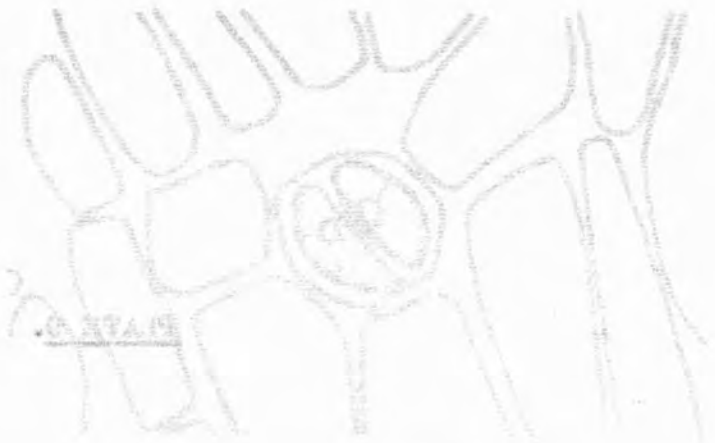
PLATE 9.

Plate 9A is a median longitudinal section of the dig-
estive gland of Dionaea, and 9 B,C, and D are
a series of transverse sections of this gland.
Fixation was in glutaraldehyde, embedding was
done in hard ester wax, and staining with
Sharman's triple stain. Drawing were made
at a magnification :-   X 500

9.
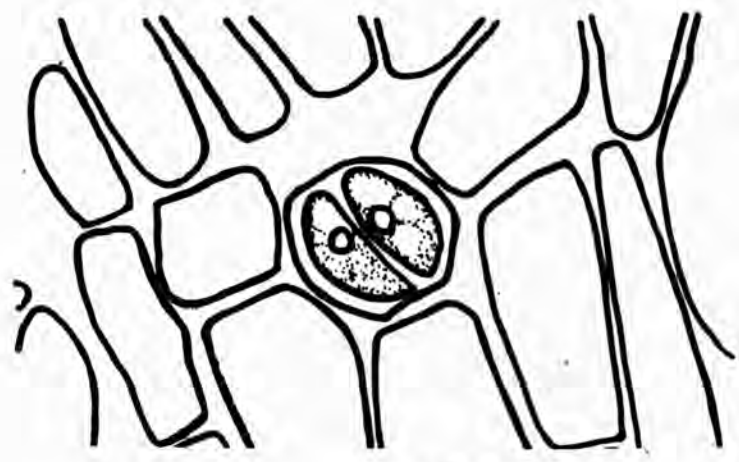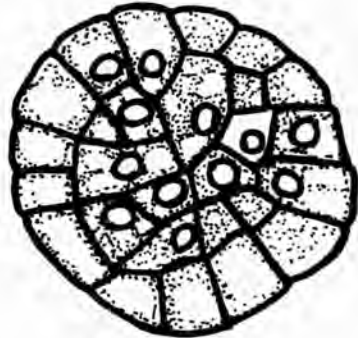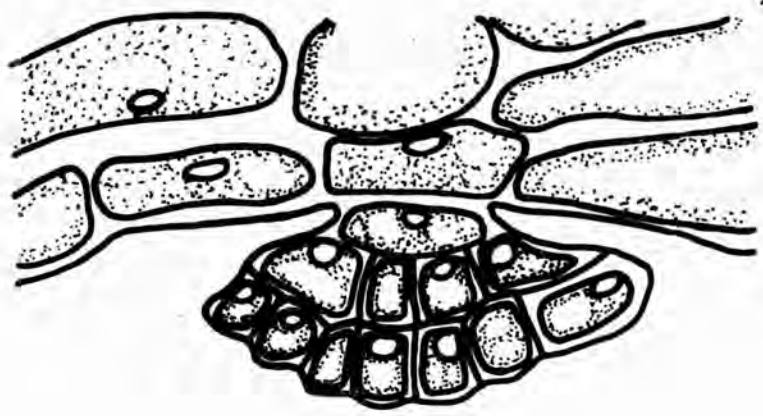
RESULTS OF HISTOCHEMICAL TEST FOR CHOLINESTERASE.

Negative results were obtained using this test. This indicated that cholinesterase was not present, and that probably acetylcholine was not present as the chemical transmitter of an action potential.

CHAPTER 6

RESULTS

ELECTRON MICROSCOPY

[...] seen at the base of the sensory hair. The type of chloroplast, mitochondria, and vesicles do not differ from those seen above and below the hinge region. The fine structure of the hinge is distinctive and different, however.

Plate 11 (pages 64 and 65) shows the mesophyll below the sensory hair of a very immature trap, about 8th the mature size. It is of interest because of the two mitochondria which are joined, and seem to have just arisen from binary fission.

Plates 12 to 21 inclusive (pages 66 to 83) show drawings made of median, longitudinal sections of the hinge region, or of

1. INTRODUCTION.

Since BENOLKEN et al (1967) showed that the sensory function is located in the "hinge" of the sensitive hair, a study of the fine structure of this region, before and after stimulation, is of interest, especially in regard to an interpretation of the mechanism involved.

The availability of an AE 1 EM 6B electron microscope, with a resolving power better than $5\overset{o}{A}$, made possible the examination of thin sections, so that an interpretation of molecular changes in membranes with stimulation could be made.

2. GENERAL DESCRIPTION OF THE FINE STRUCTURE OF THE SENSORY HAIR AND ITS ORGANELLES.

Plate 10 (pages 62 and 63) shows typical mesophyll tissue as seen at the base of the sensory hair. The type of chloroplast, mitochondria, and vesicles do not differ from those seen above and below the hinge region. The fine structure of the hinge is distinctive and different, however.

Plate 11 (pages 64 and 65) shows the mesophyll below the sensory hair of a very immature trap, about ⅛th the mature size. It is of interest because of the two mitochondria which are joined, and seem to have just arisen from binary fission.

Plates 12 to 21 inclusive (pages 66 to 85) show drawings made of median, longitudinal sections of the hinge region, or of

portions of the region. (For interpretation, see KEY DIAGRAMS

and ABBREVIATIONS immediately following the index.) In most cases

the fixative used was glutaraldehyde, followed by osmic acid, but

Luft's permanganate was also used. Such drawings made possible a

much more accurate count, and study of organelles and fine struc-

ture (e.g., in counting mitochondria swollen following stimulation).

Plates 22 to 40 inclusive (pages 86 to 122) show representative

low and high power photographs of the unstimulated hinge region in

MLS, whilst plate 53, page 148, shows a Golgi apparatus, X 60,000,

in an unstimulated hinge cell, where permanganate was used as the

fixative.

Plates 41 to 52 inclusive (pages 123 to 146) show representative

low and high power photographs of the MLS of the sensory hinge,

when fixed in the bent position.

(A). LIGHT MICROSCOPY.

EXPLANATION OF PLATES.

AT- artifact
CH- chloroplast
CU- cuticle
CW- cell wall
G - Golgi apparatus
ER- endoplasmic reticulum
M - mitochondrion
ML- middle lamella
MT- microtubule
N - nucleus
NM- nuclear membrane

NU - nucleolus
O - oil droplet
P - pore
PD - plasmodesmata
PL - plastid
PM - plasma membrane
PS - pore site
T - tonoplast
V - vacuole
VE - vesicle

EXPLANATION OF DIAGRAMS SHOWN IN PLATES.

Endoplasmic reticulum - nearly parallel lines
Golgi apparatus   - as would appear in actual electron
                           micrographs
Nucleus   - solid black
Nucleolus   - white
Plastids   - organelles with internal lines
Vesicles   - very small circles.

HINGE CELL

(A). LIGHT MICROSCOPY.

ML
CU
CW
CY
V
N

(B) ELECTRON MICROSCOPY.

ER
CU
CW
CY
V
N
NU
M
G
G
PL

VE — ML

CONDUCTING
CELL

HINGE CELL
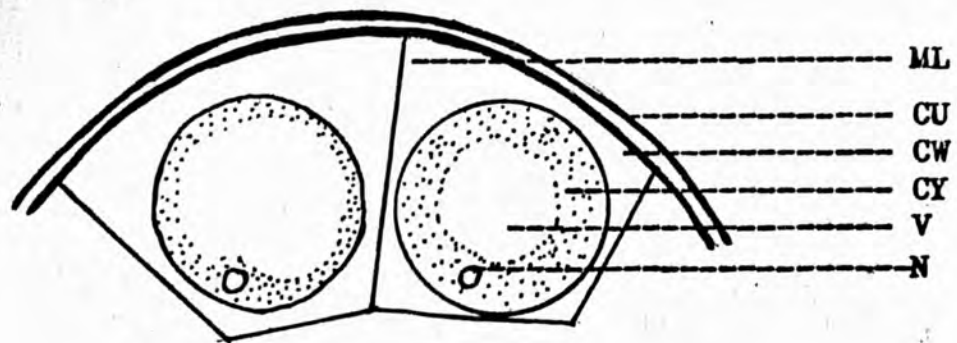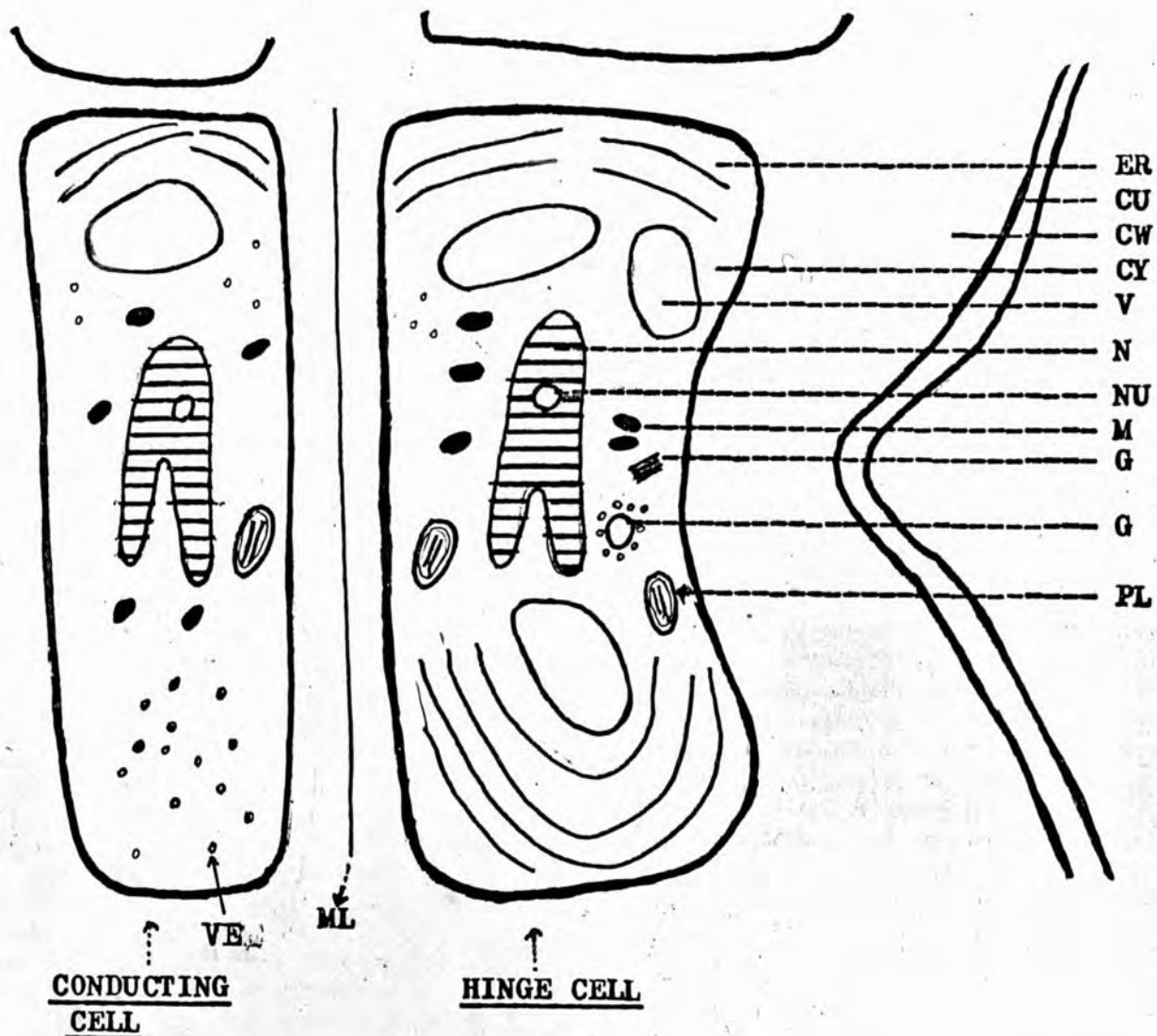
1 B KEY DRAWINGS TO ILLUSTRATE DIAGRAMS SHOWN IN PLATES.

PLATE 10.

A section through the mesophyll of the
trap of Dionaea. Fixed in Luft's
permanganate, embedded in araldite and
stained with Reynold's lead citrate.

X 23000

PLATE 10.

A section through the mesophyll of the
trap of Dionaea. Fixed in Luft's
permanganate, embedded in araldite and
stained with Reynold's lead citrate.

X 23000

10

63

PLATE II

Section through the young mesophyll of an
immature trap of Dionaea. Fixed in
glutaraldehyde, post-fixed in osmic acid,
stained with uranyl acetate and lead
citrate.

Note the mitochondria, apparently
dividing.

X 9000.

PLATE 11

Section through the young mesophyll of an

immature trap of Dionaea. Fixed in

glutaraldehyde, post-fixed in osmic acid,

stained with uranyl acetate and lead

citrate.

Note the mitochondria, apparently

dividing.

X 5000.

PLATE 12

Drawing made from high power photographs of
the median longitudinal section of the sensory
hinge.

Unstimulated.

Glutaraldehyde-osmic acid fixation.

PLATE   12

Drawing made from high power photographs of
the median longitudinal section of the sensory
hinge.

Unstimulated.

Glutaraldehyde-osmic acid fixation.

12

20μ

PLATE 13

A drawing made from high power E.M.

photographs of the median longitudinal

section of the sensory hair hinge.

Unstimulated.

Permanganate fixation,
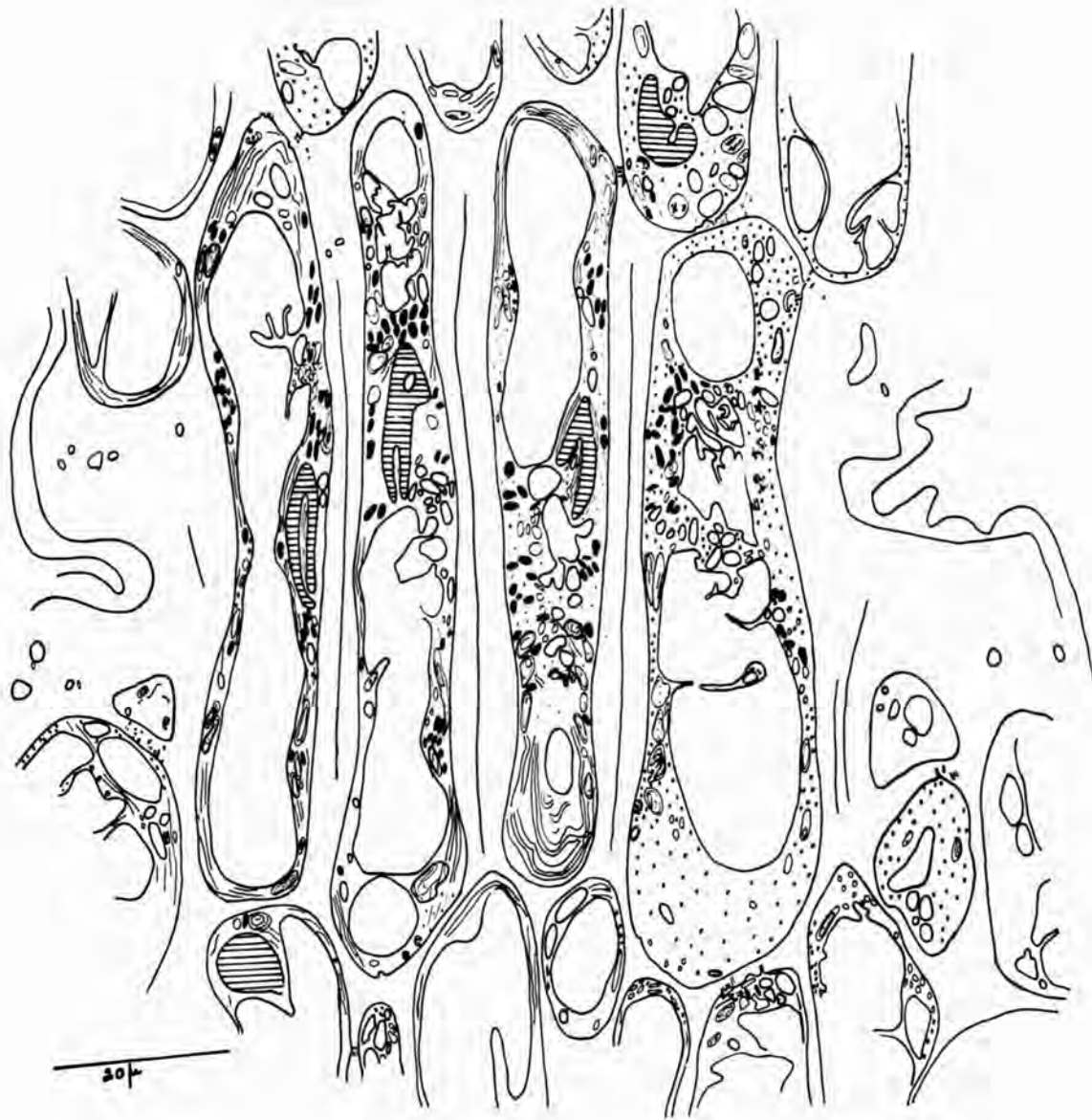
Uranyl acetate and lead citrate staining.

X 2500.

PLATE 13

A drawing made from high power E.M.

photographs of the median longitudinal

section of the sensory hair hinge.

Unstimulated.

Permanganate fixation,

Uranyl acetate and lead citrate staining.

X   2500.

13
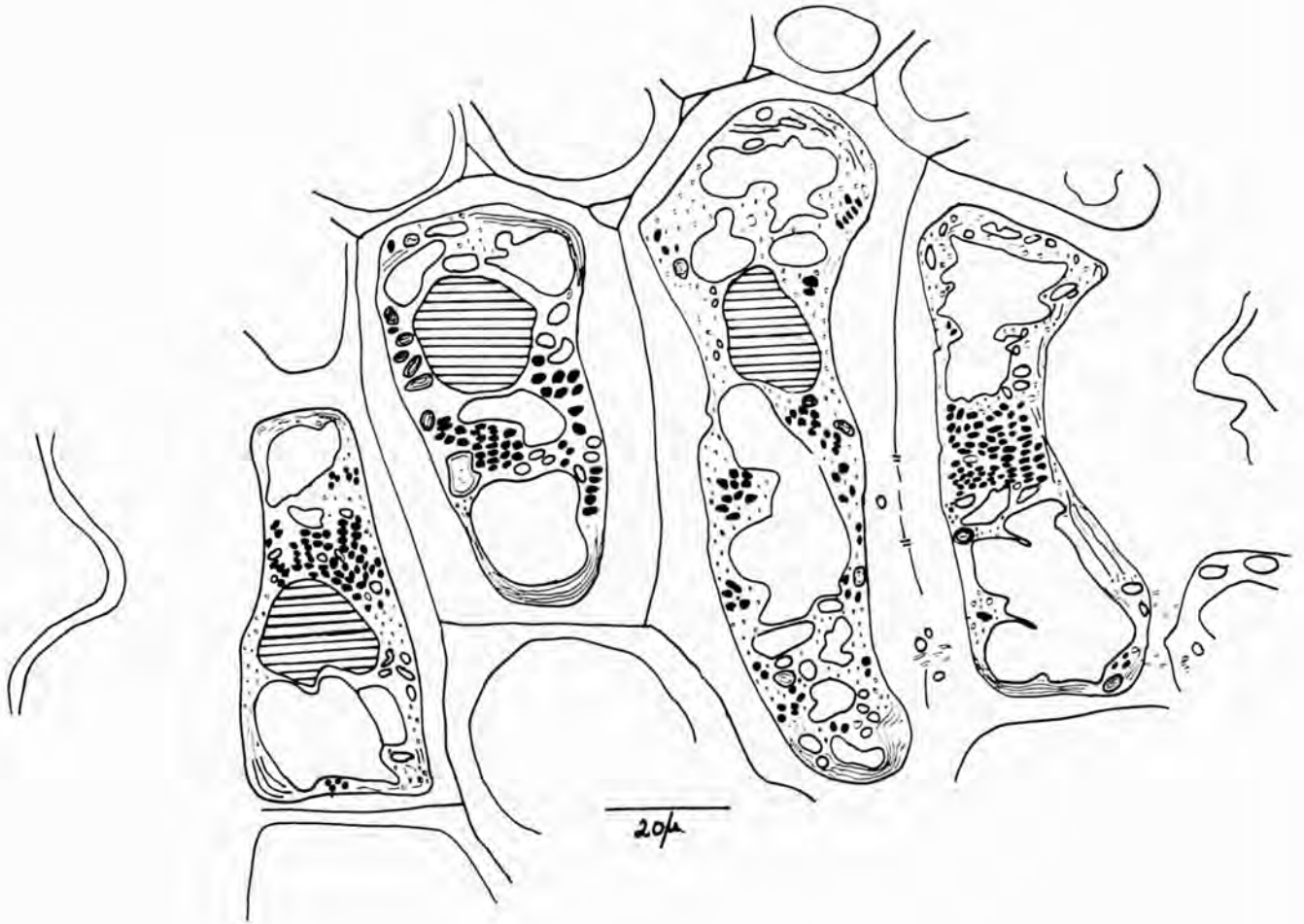
20μ

PLATE 1A

Drawing made from high power electron microscope photographs of the median longitudinal section of the sensory hair hinge.

Unstimulated.

Glutaraldehyde fixation,

Osmic acid post-fixation,

Uranyl acetate & lead citrate staining.

X 2500.

PLATE 14

Drawing made from high power electron

microscope photographs of the median

longitudinal section of the sensory

hair hinge.

Unstimulated.

Glutaraldehyde fixation,

Osmic acid post-fixation,

Uranyl acetate & lead citrate staining.

X 2500.

14

20 μ
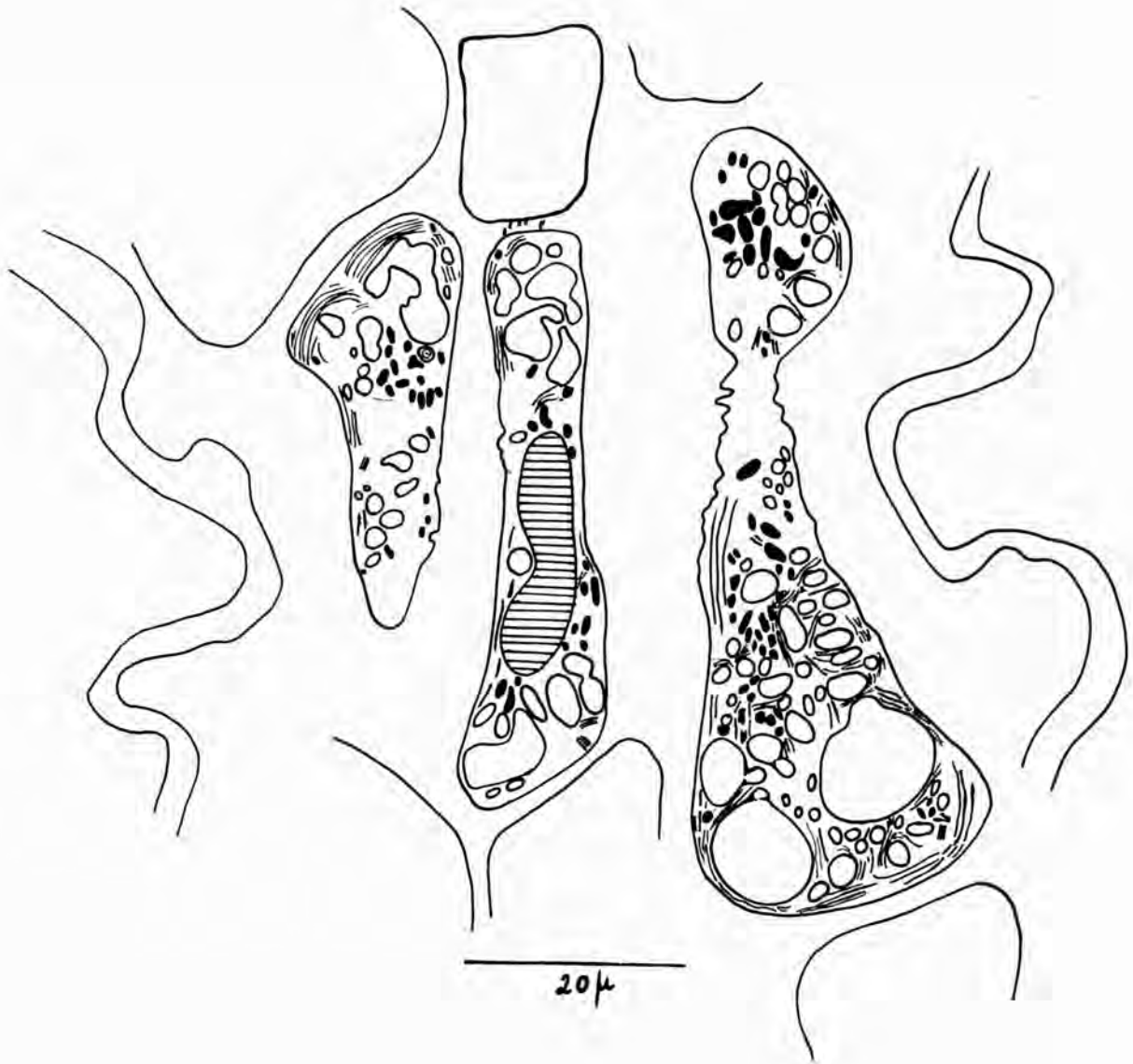
PLATE 15

Drawing made from high power, electron

microscope photographs of a median,

longitudinal section of portion of the

sensory hair hinge of Dionaea.

Permanganate fixation.

Unstimulated.

X 5000.

10μ

PLATE 16

Drawing from high power, electron
microscope photographs of portion of
the hinge region of the sensory hair
of Diapasa. Permanganate fixation.
Unstimulated.
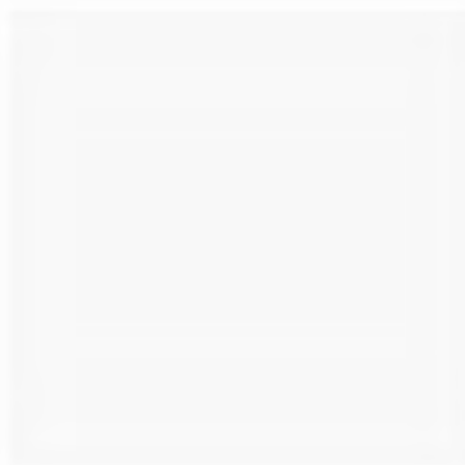
X 2600

PLATE 16

Drawing from high power, electron
microscope photographs of portion of
the hinge region of the sensory hair
of Dionaea. Permanganate fixation.
Unstimulated.

X 2600

20/ι
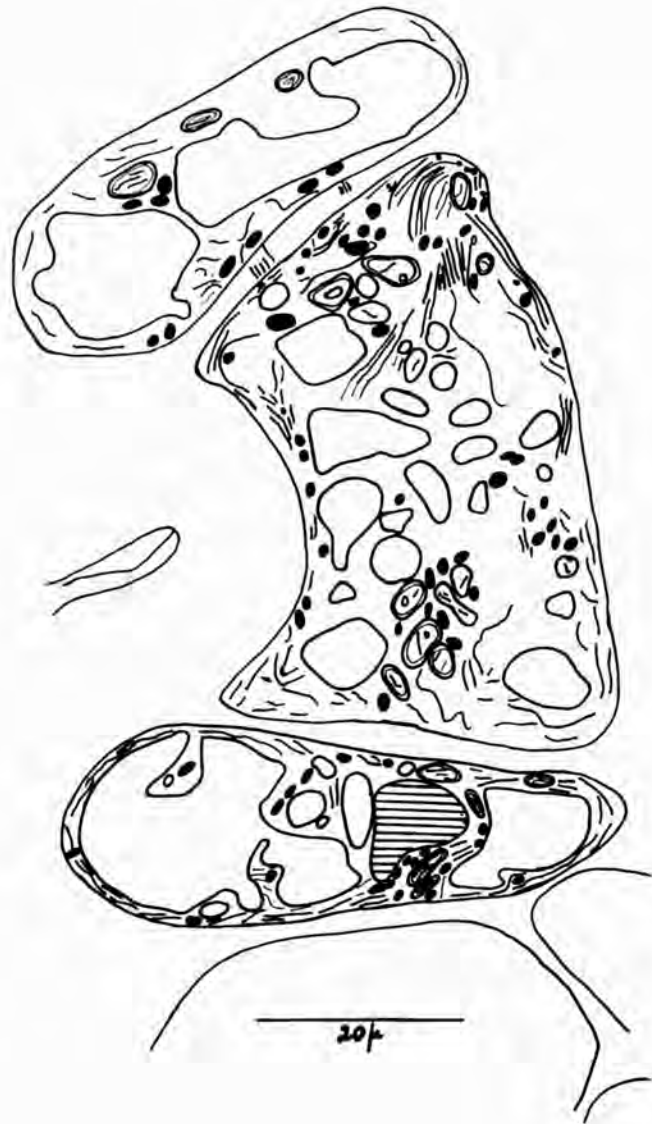
PLATE 17

Drawing made from high power

photographs of sensory hair

hinge. Median L.S. Hair

stimulated - fixed in the bent

position. Glutaraldehyde fixation,

osmic acid post-fixation.

X 1300

17
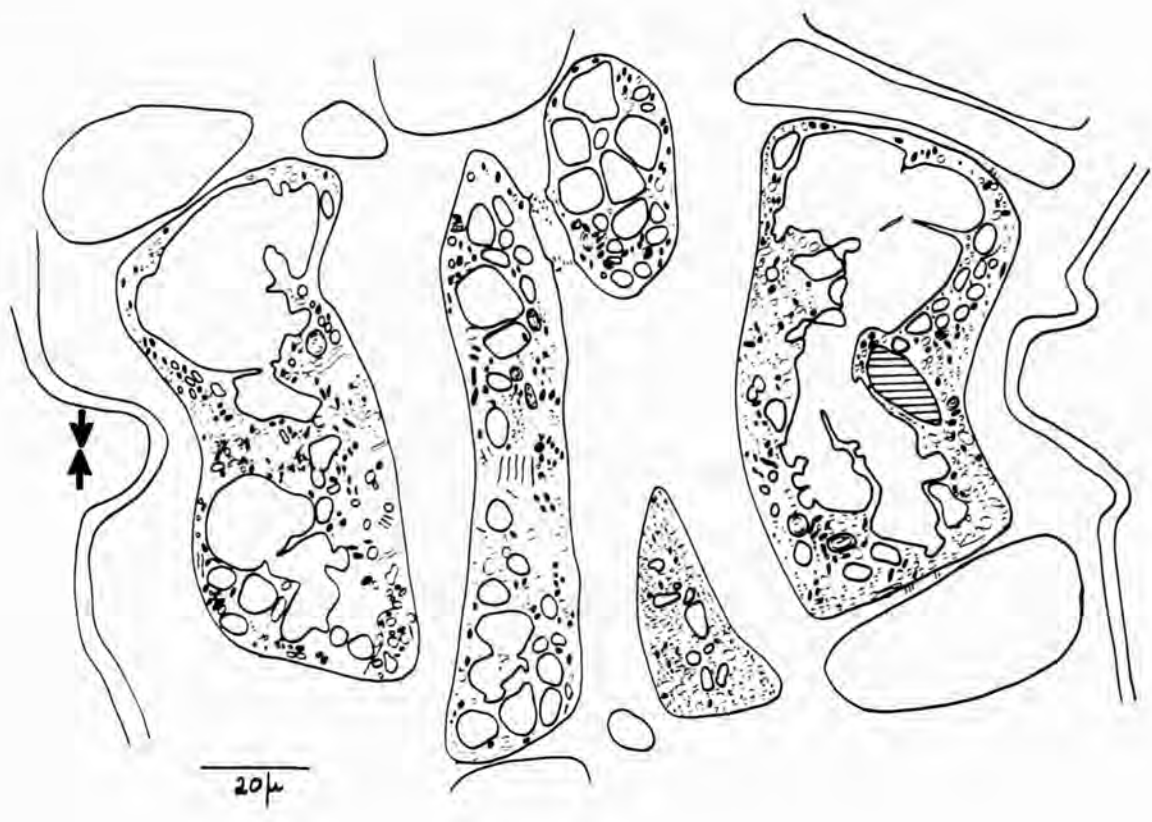
20μ

PLATE 18

Drawings made from high power electron
microscope photographs of the sensory
hair cells. Median longitudinal section.
Stimulated by bending. Fixed in the
bent position with glutaraldehyde, and
post-fixed with osmic acid.
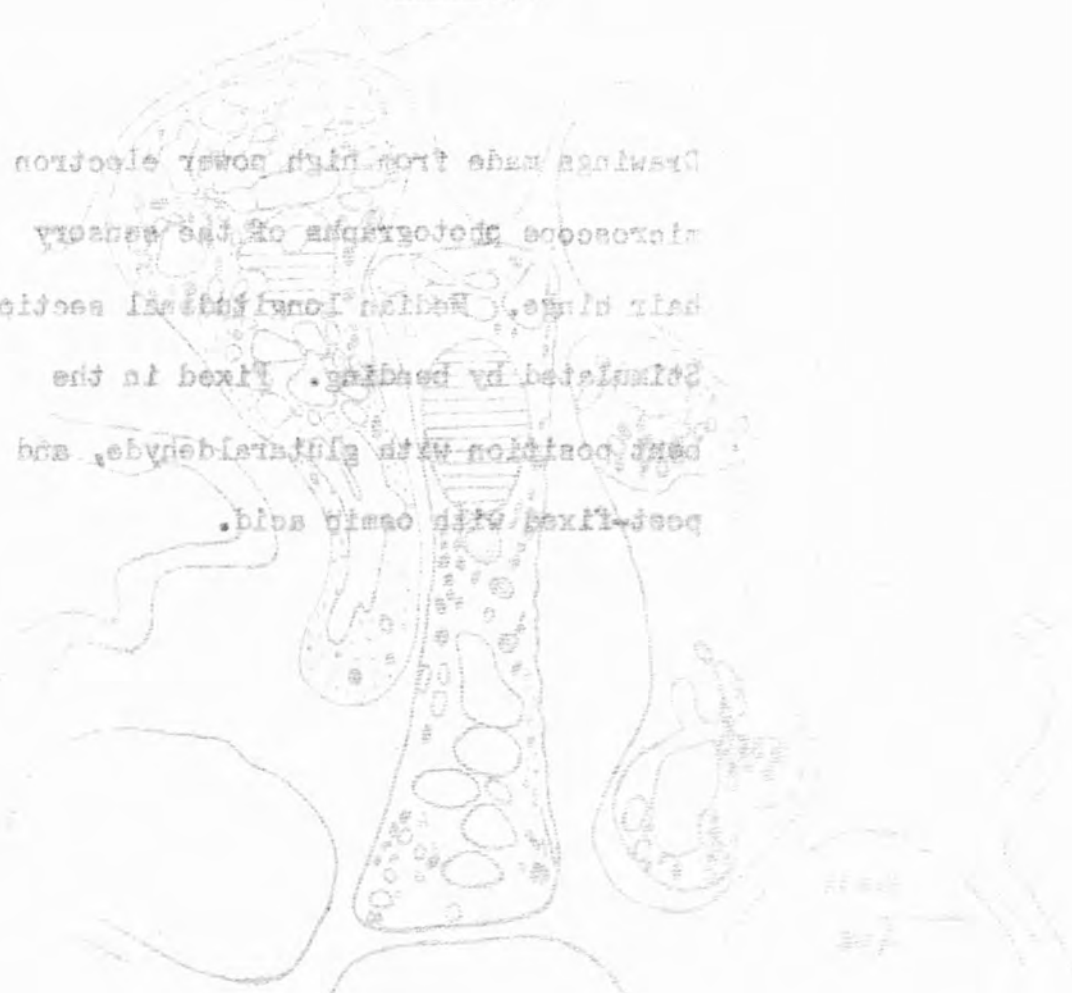
PLATE 18

Drawings made from high power electron

microscope photographs of the sensory

hair hinge. Median longitudinal section.

Stimulated by bending. Fixed in the

bent position with glutaraldehyde, and

post-fixed with osmic acid.

18

SCALE

20 µ
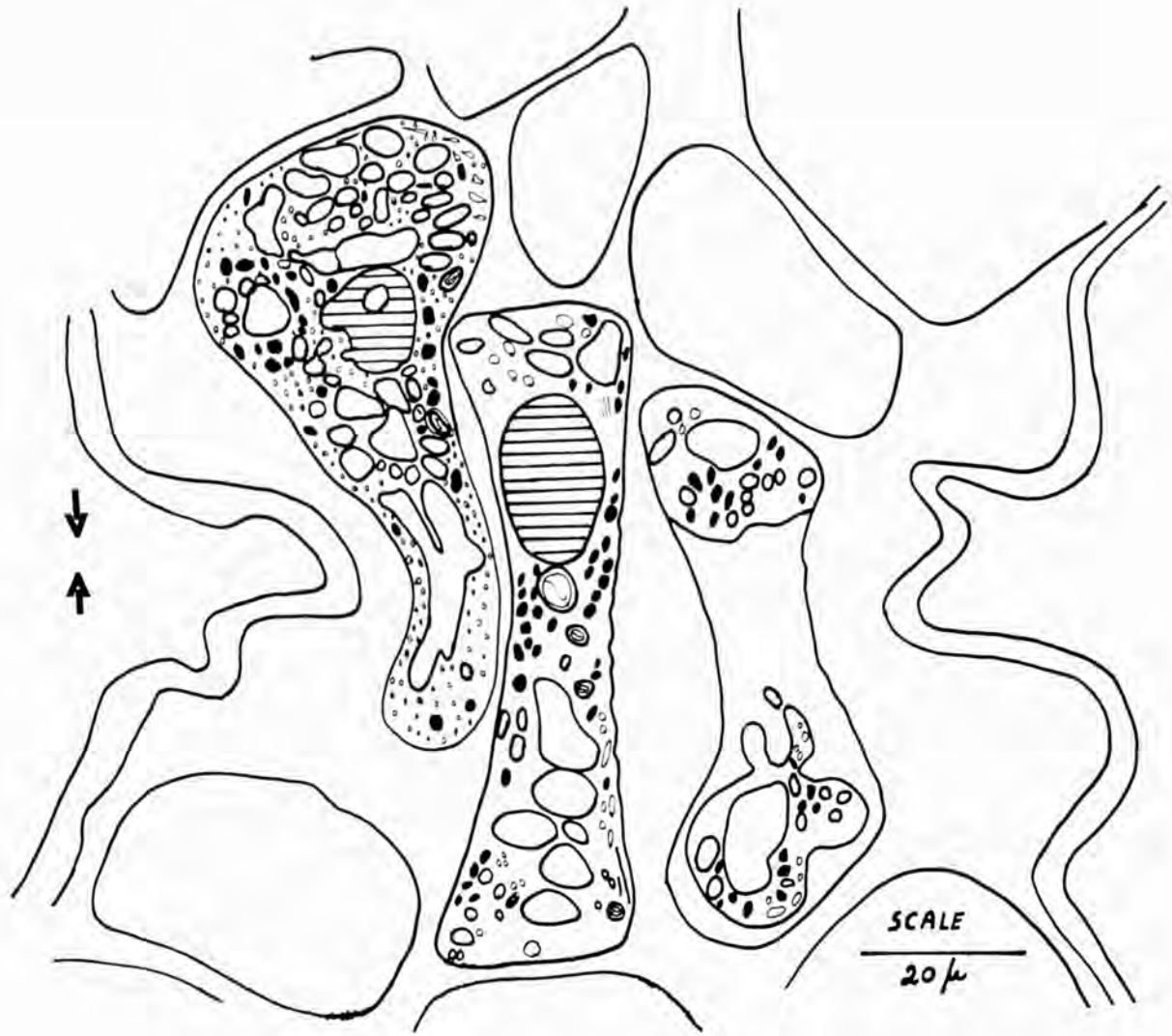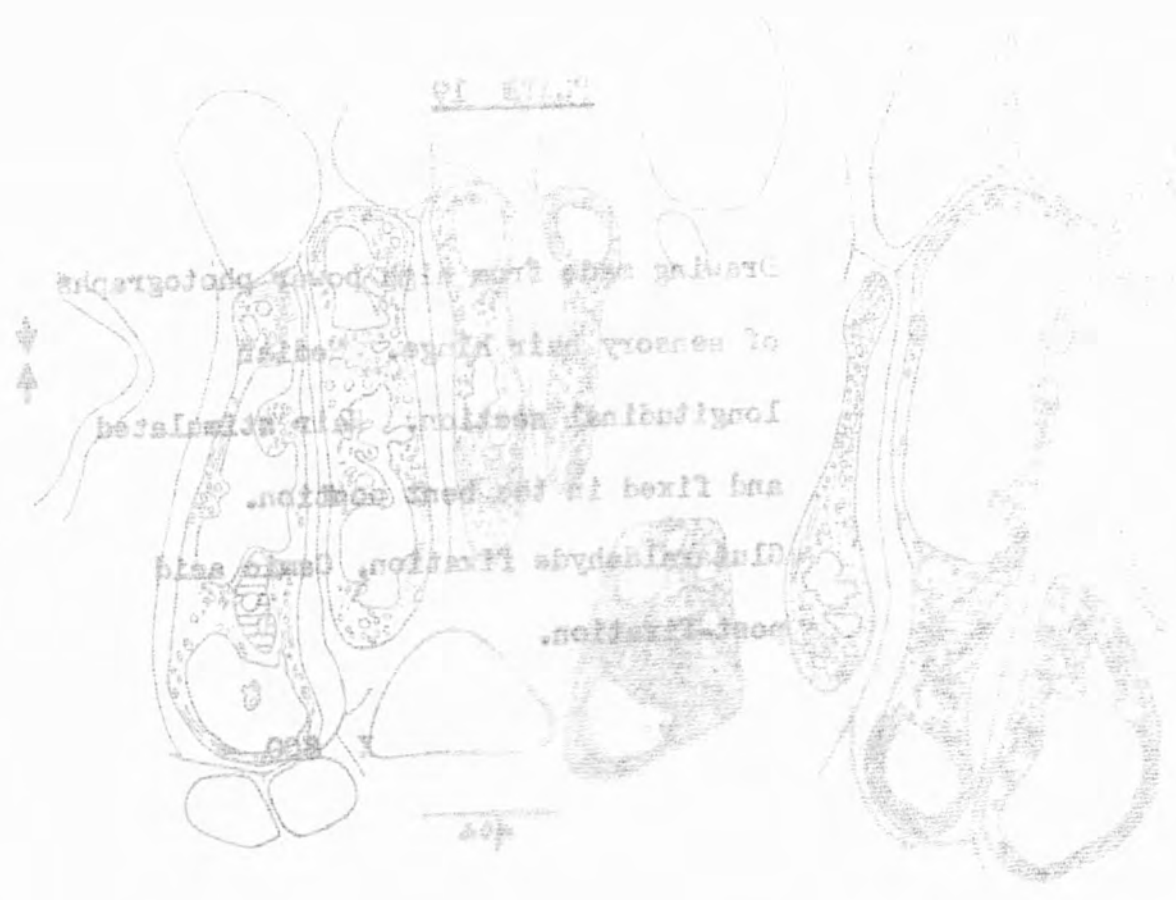
PLATE 19

Drawing made from high power photographs

of sensory hair hinge. Median

longitudinal section. Hair stimulated

and fixed in the bent position.

Glutaraldehyde fixation, Osmic acid

post-fixation.

X 800

19
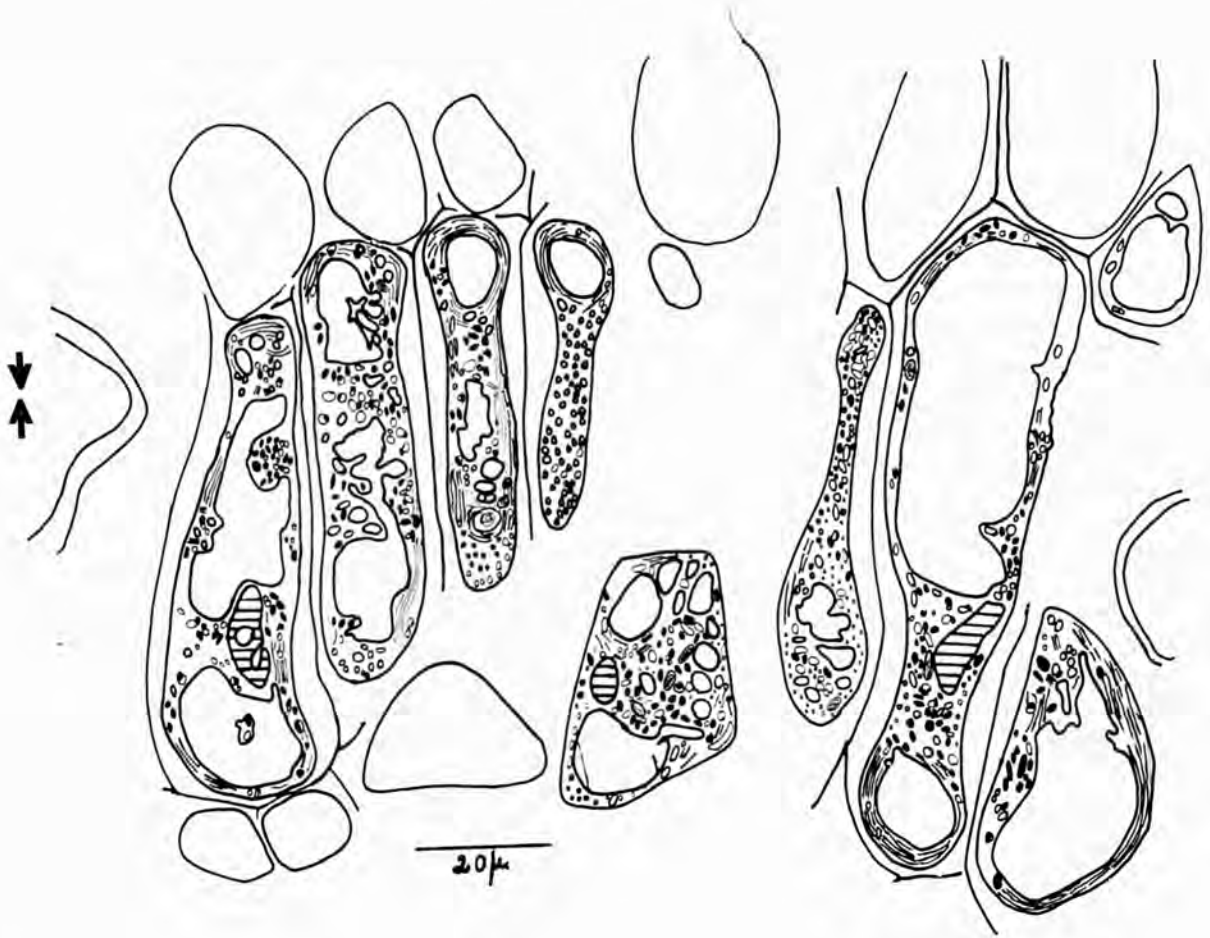
20 μ

PLATE 20.

Drawing made of sensory hair hinge cell,

fixed in the bent position with glutaraldehyde,

and post-fixed with osmic acid.

Stimulated.
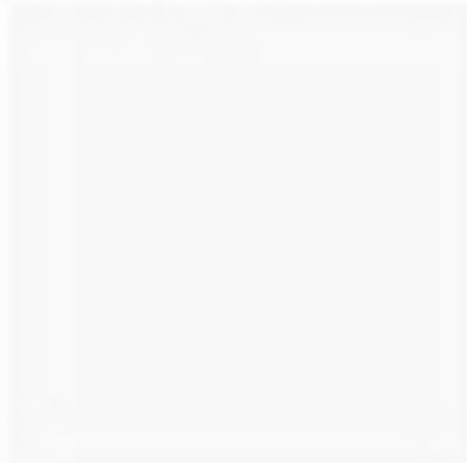
20
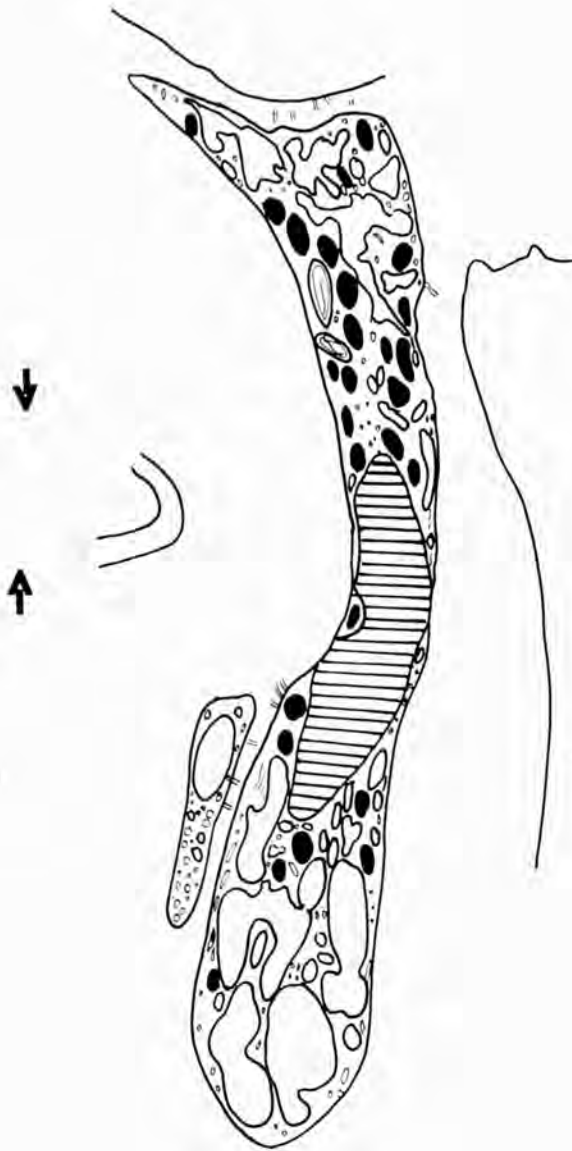
5 μ

FIGURE 24.

...raws is made from high power

...photographs of sensory hair discs.

...Median longitudinal section.

...air stimulated, and fixed

...in the bent position.

Glutaraldehyde fixation.

...mic acid post-fixation.
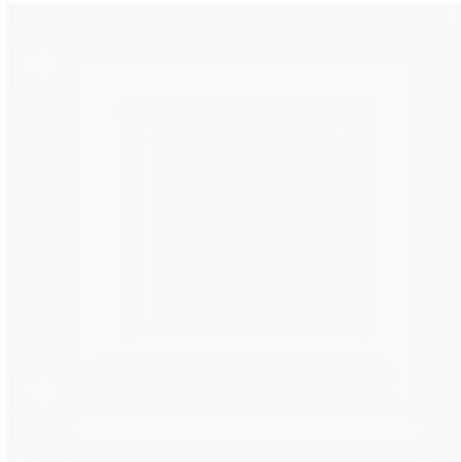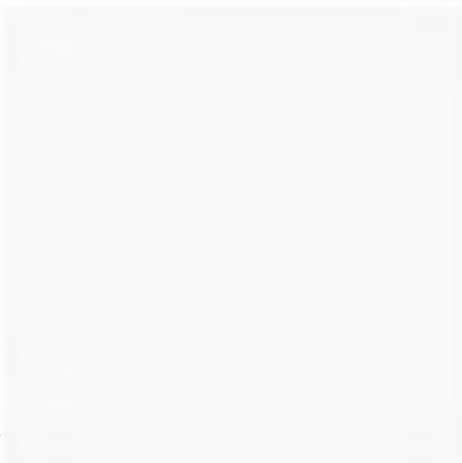
PLATE 21.

Drawing made from high power

photographs of sensory hair hinge.

Median longitudinal section.

Hair stimulated, and fixed

in the bent position.

Glutaraldehyde fixation.

Osmic acid post-fixation.

21
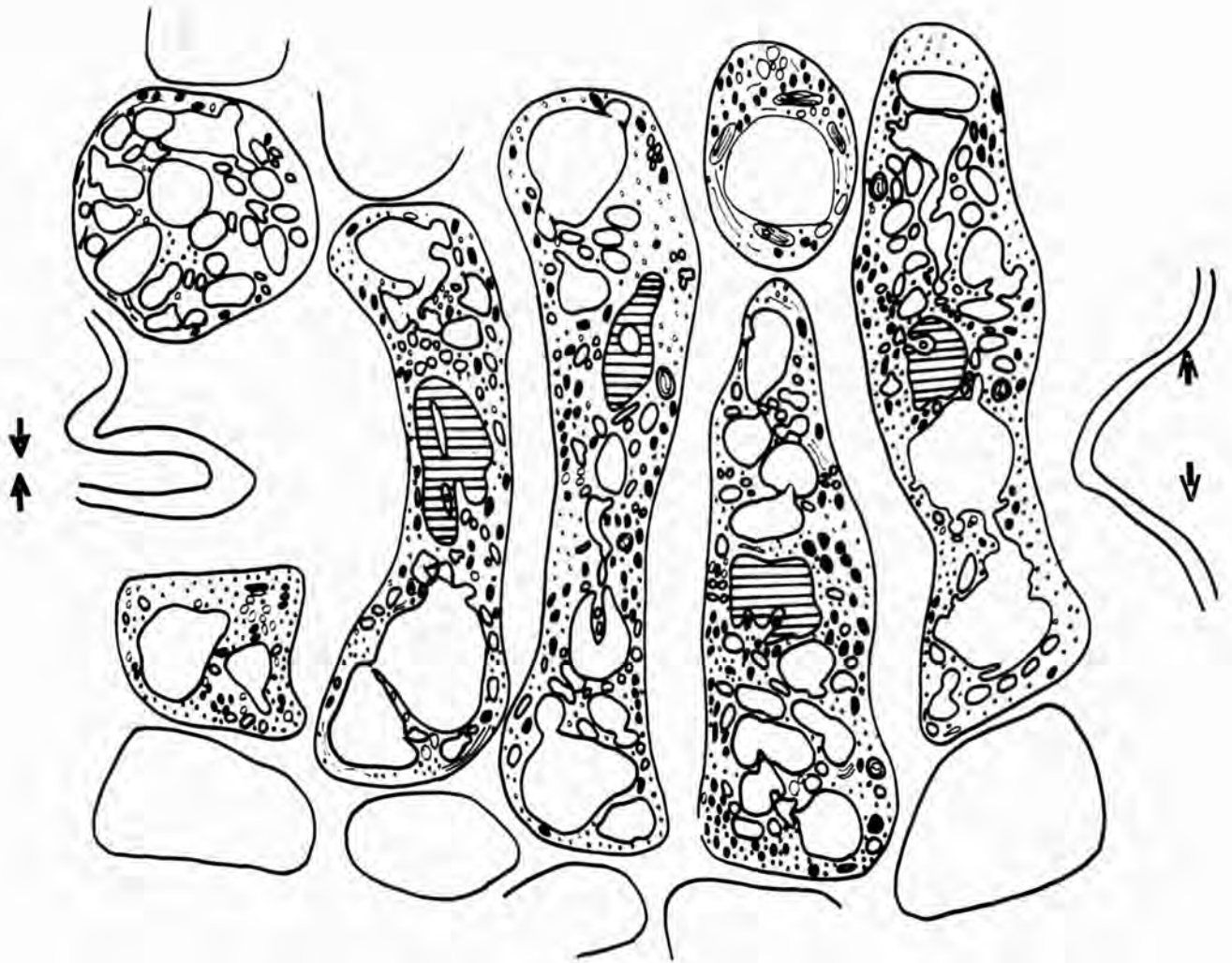
20 μ

PLATE 22.

Huge cell of sensory hair.
Permanganate fixed.
This cell is represented as part of
the drawing of plate 13.
Unstimulated. Note the
numerous mitochondria clustered
near the centre, extensive ER,
vesicles and vacuoles, thick walls
and plastids.

X 6,000.

PLATE 22.

Hinge cell of sensory hair.

Permanganate fixed.

This cell is represented as part of

the drawing of plate 13.

Unstimulated. Note the

numerous mitochondria clustered

near the centre, extensive ER,

vesicles and vacuoles, thick walls

and plastids.

X 6,000.

22
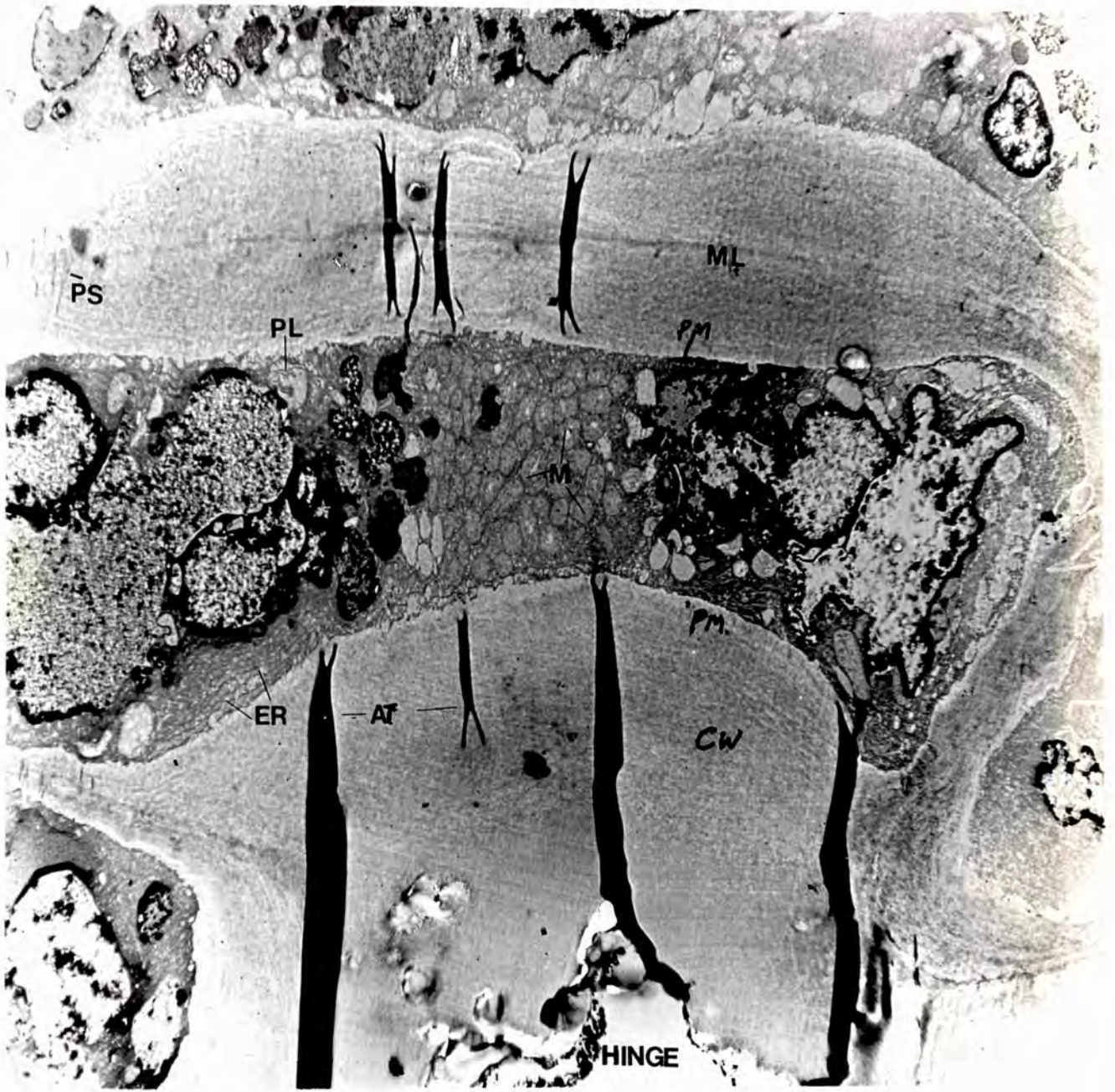
PLATE 23

Median longitudinal section of
sensory hair hinge, represented
in the drawing shown in plate 12.
Unstimulated. Fixed in glutaraldehyde,
and post-fixed in osmic acid.
Stained with uranyl acetate-lead citrate.
Note the lobed nuclei, the single
nucleolus, numerous vesicles, the
organelles clustered near the centre,
large vacuoles and extensive E.R.

X   2000.
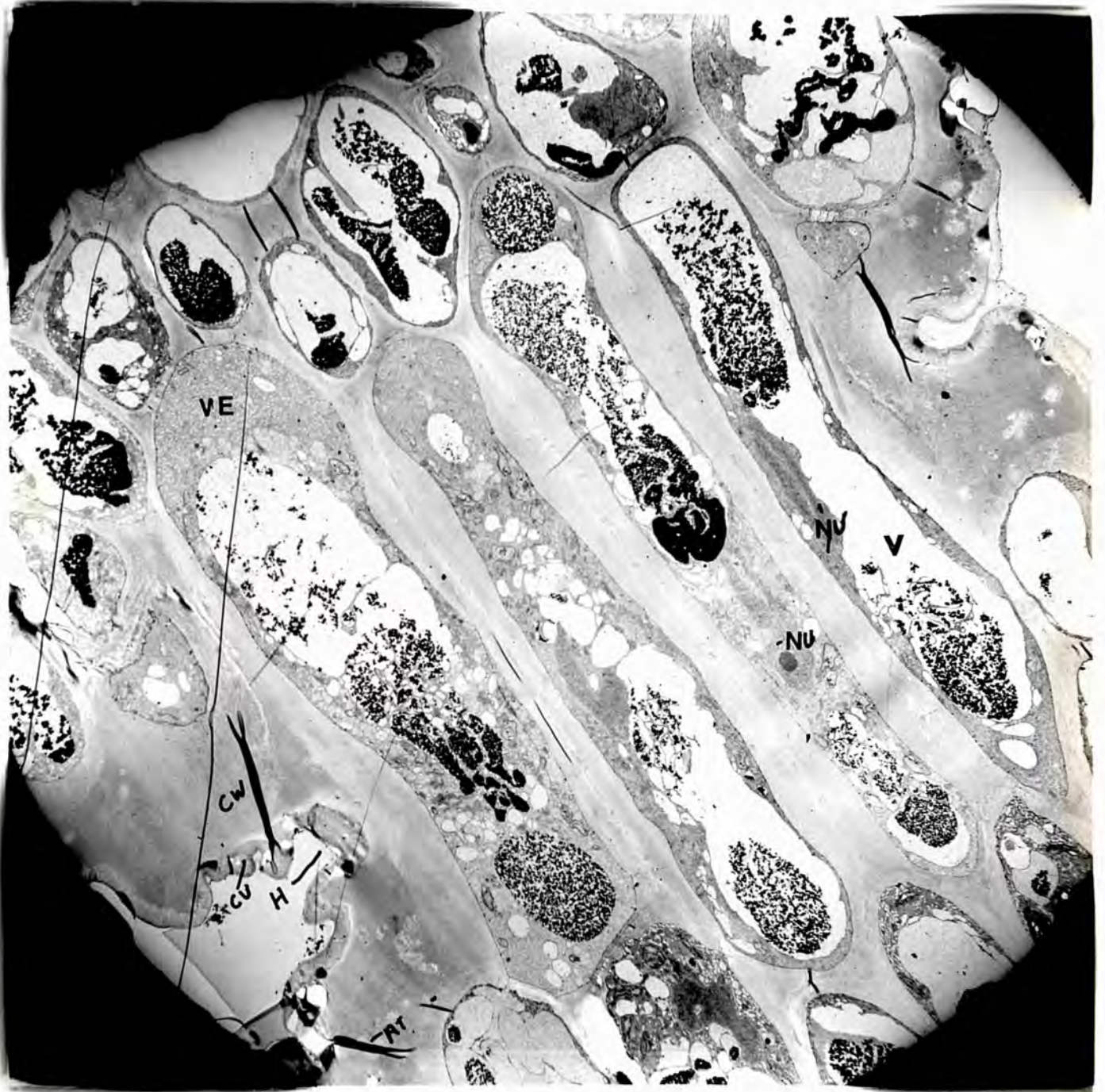
PLATE 24

This is a portion of the same section as shown in plates 12 and 23, but at a higher magnification.

ote the numerous vacuoles, vesicles and oil droplets, and the extensive endoplasmic reticulum.

X 5000.

PLATE 24

This is a portion of the same section
as shown in plates 12 and 23, but at
a higher magnification.

Note the numerous vacuoles, vesicles
and oil droplets, and the extensive
endoplasmic reticulum.

X 5000.

PLATE 24

This is a photograph of portion of a cell, shown in plate 24, and taken at a higher magnification, to illustrate the extensive endoplasmic reticulum, plastids, Golgi, lipid droplets and numerous vesicles.

X 15000.

PLATE 25

This is a photograph of portion of a cell
shown in plate 24, and taken at a higher
magnification, to illustrate the
extensive endoplasmic reticulum, plastids,
Golgi, lipid droplets and numerous vesicles.

X 15000.
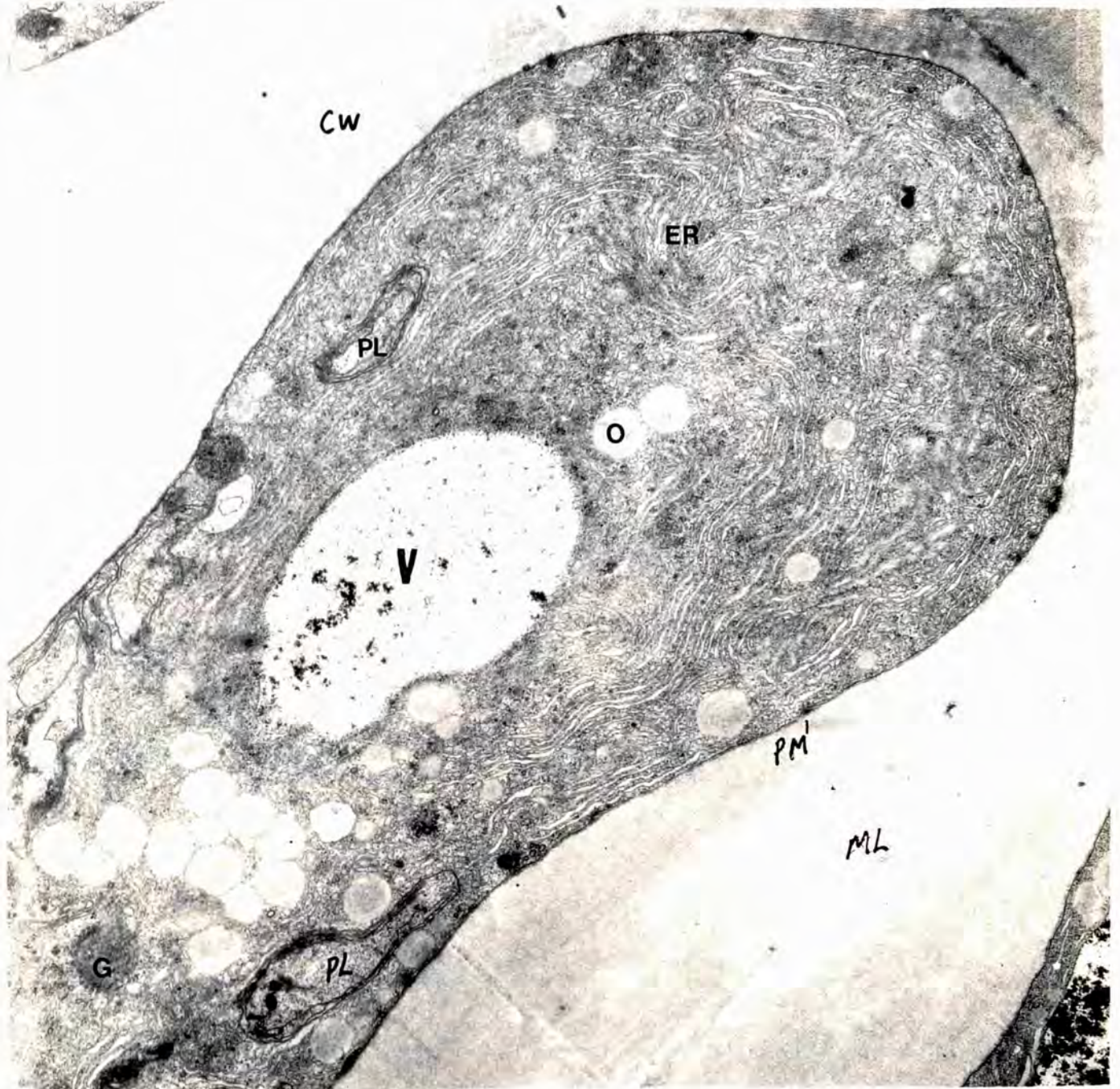
PLATE 26

Photograph taken at a higher magnification,
adjacent to and overlapping portion of
plate 25. This plate illustrates Golgi,
vesicle formation, mitochendria and
plastids.

X 40,000.
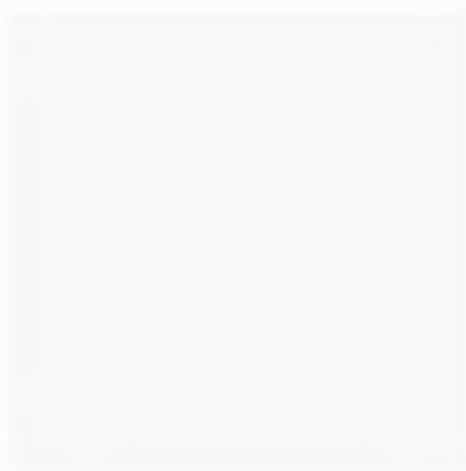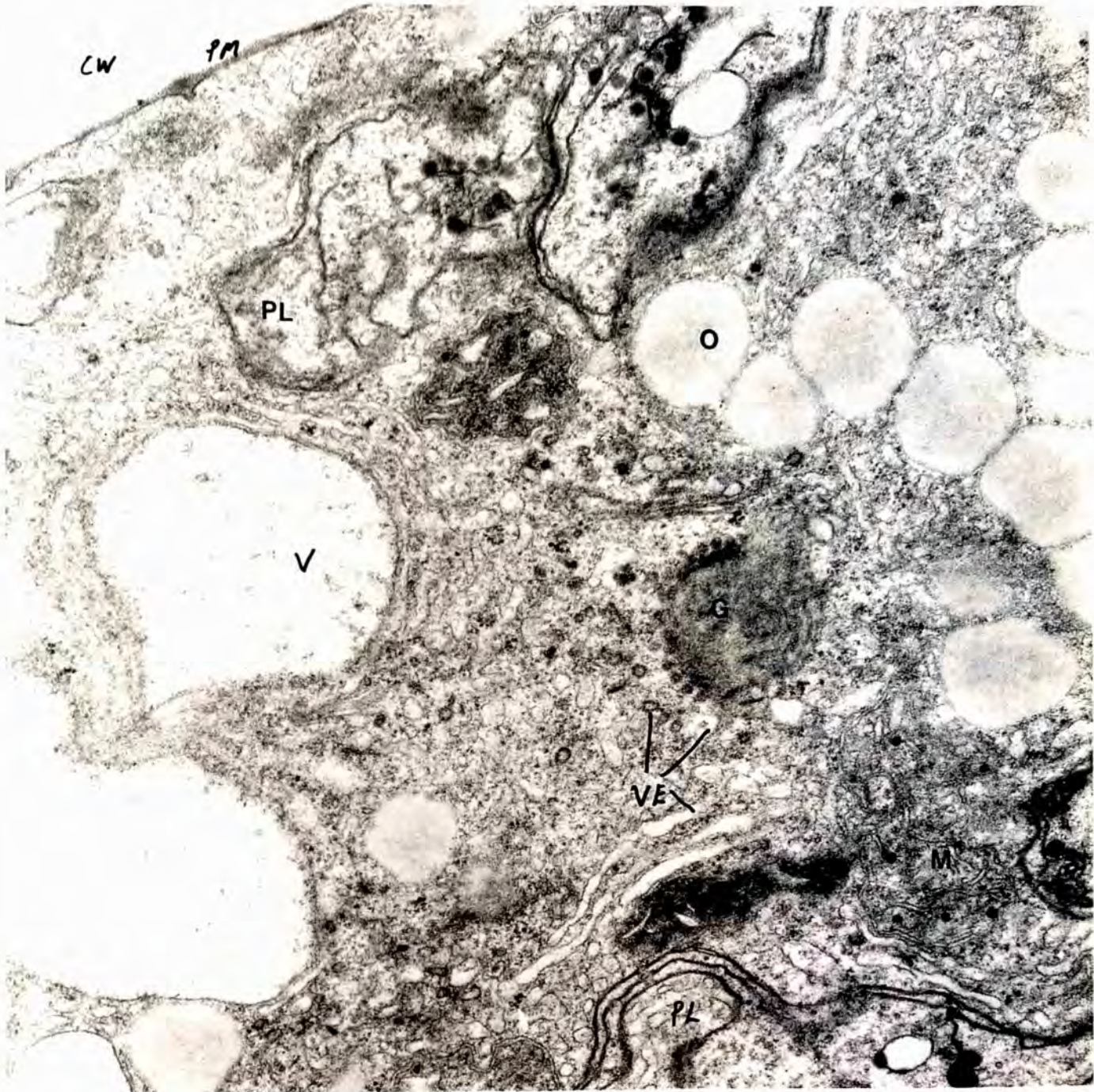
26

Figure 27

...ortion near the central region of a
sensory hinge cell shown in plate 27,
but at a higher magnification. This
illustrates the mitochondria crowded
near the centre, plastids, Golgi,
vesicles, and large vacuoles with
deposits inside.

X 20,000.
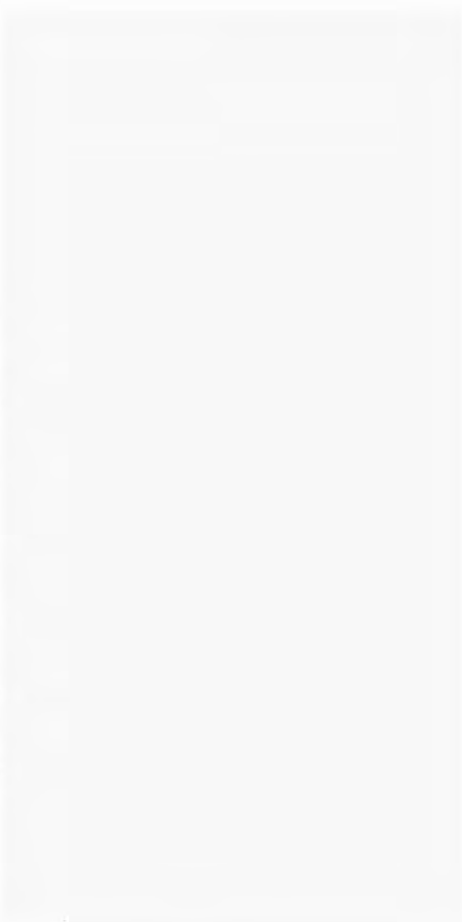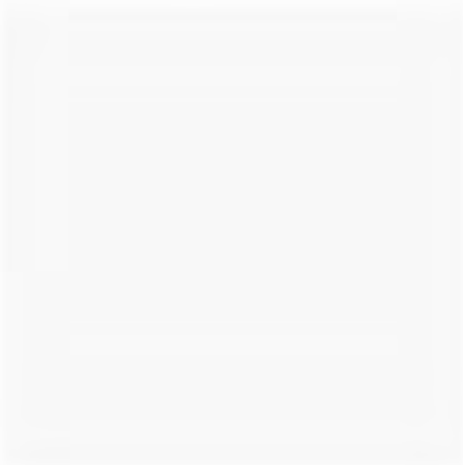
PLATE   27

Portion near the central region of a
sensory hinge cell shown in plate 23,
but at a higher magnification. This
illustrates the mitochondria crowded
near the centre, plastids, Golgi,
vesicles, and large vacuoles with
deposits inside.

X 20,000.

27
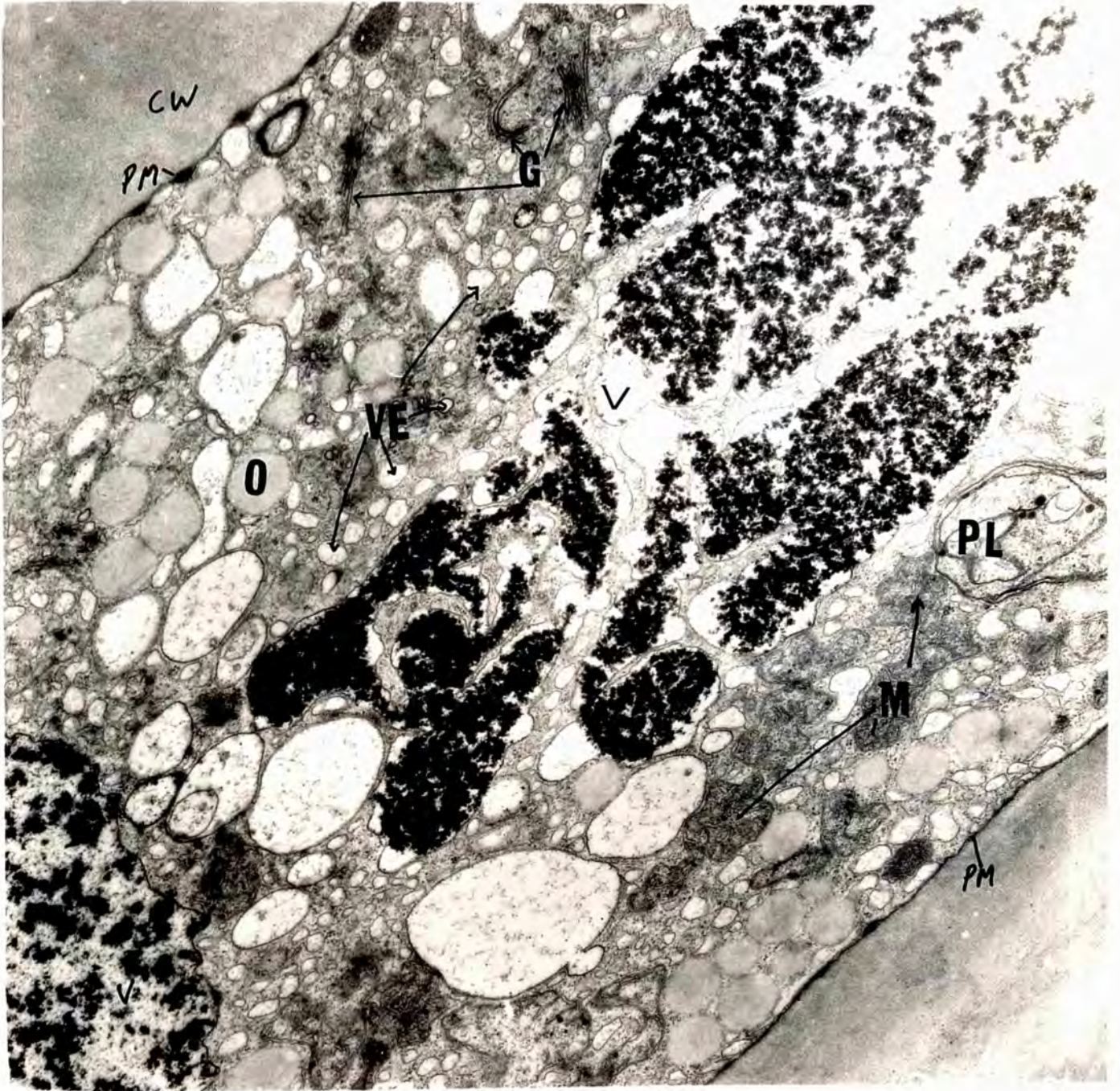
Plate 23

Photograph at high magnification of a portion of a large cell of the ... hair shown in plate 23, in order to show structure of the plastid, mitochondria, Golgi and vacuoles.

X 30,000.

PLATE 28

A photograph at high magnification

of portion of a hinge cell of the

sensory hair shown in plate 23, in

order to show structure of the

plastid, mitochondria, Golgi and

vesicles.

X 50,000.
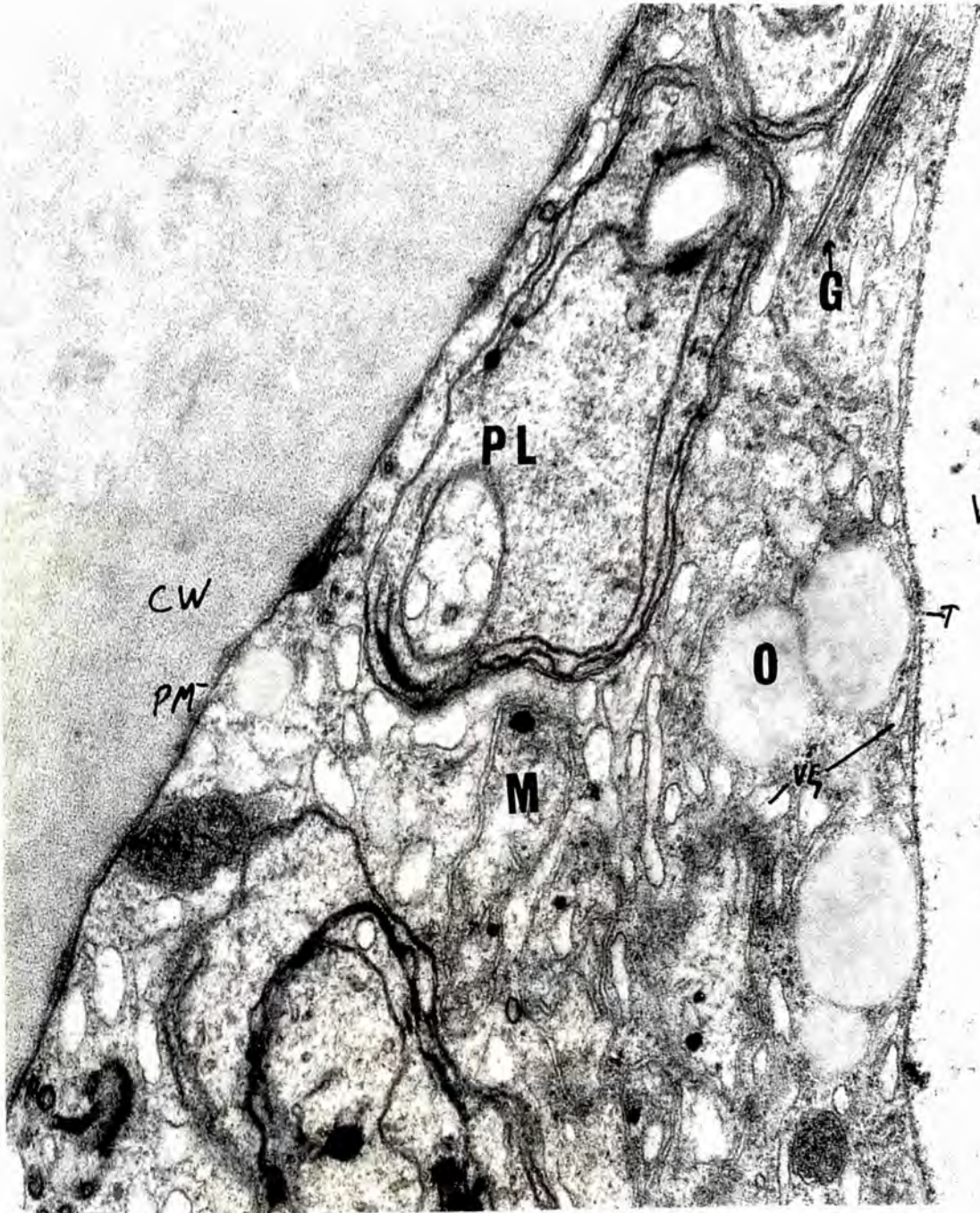
28

-100-

PLATE 29

High power electron microscope photograph near the centre of plate 23, in one of Darwin's "conducting cells" of the sensory hinge, showing the nucleus, extensive vesicularisation, and three lipid droplets.

X 60,000.

PLATE 29

High power electron microscope photograph
near the centre of plate 23, in one of
Darwin's "conducting cells" of the
sensory hinge, showing the nucleus,
extensive vesicularisation, and three
lipid droplets.

X 60,000.

29
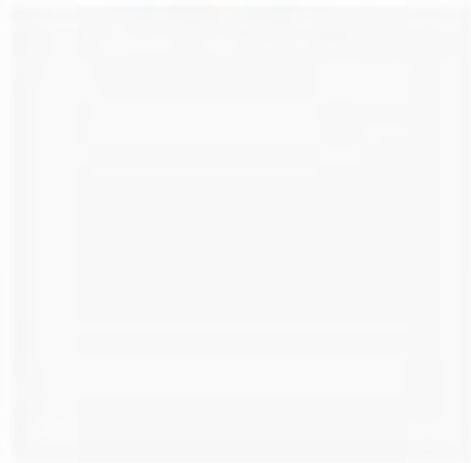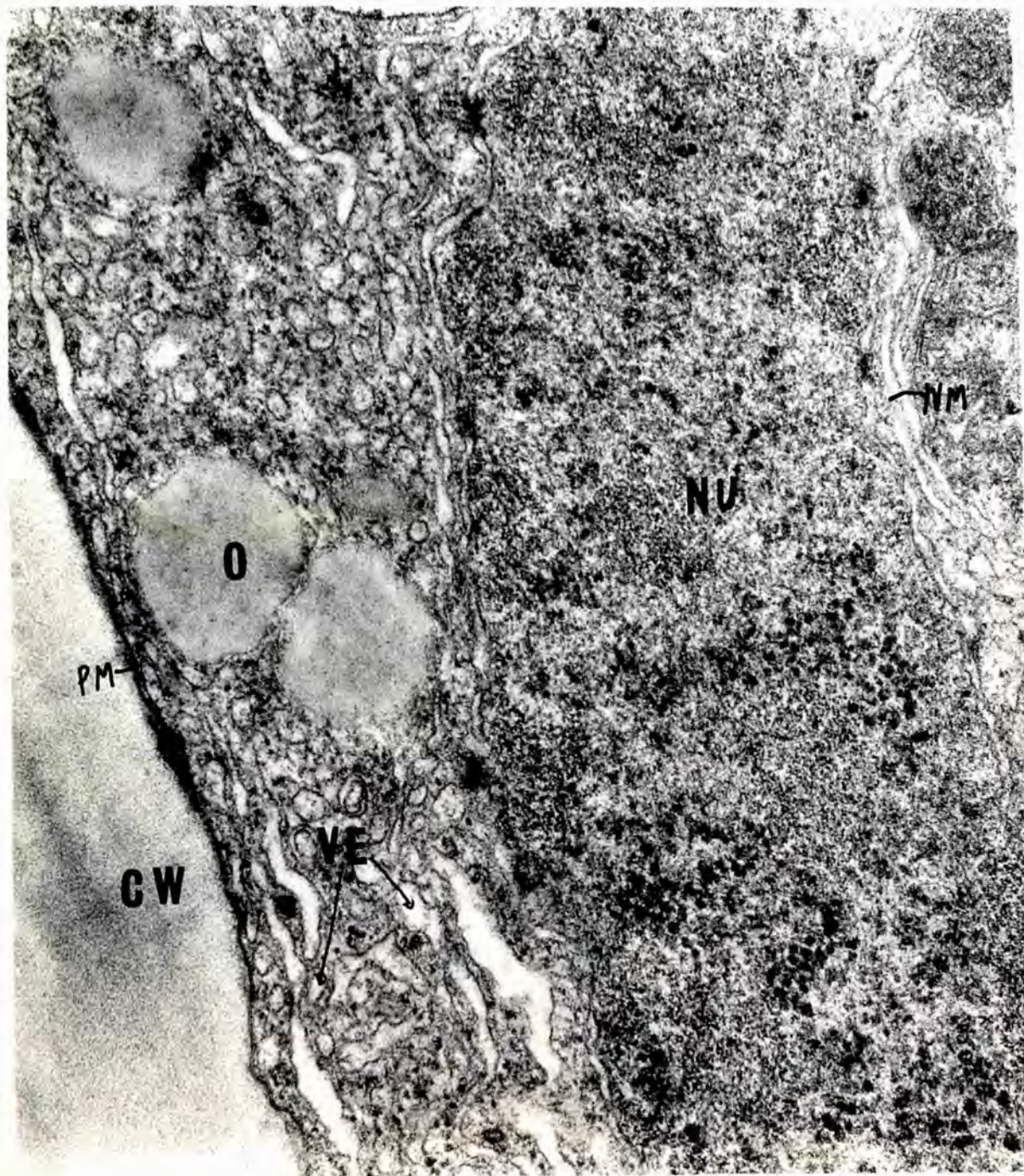
PLATE 30.

A photograph of a plasmodesma.
communicating between a "hinge
cell" and a central cell of the
sensory hinge.
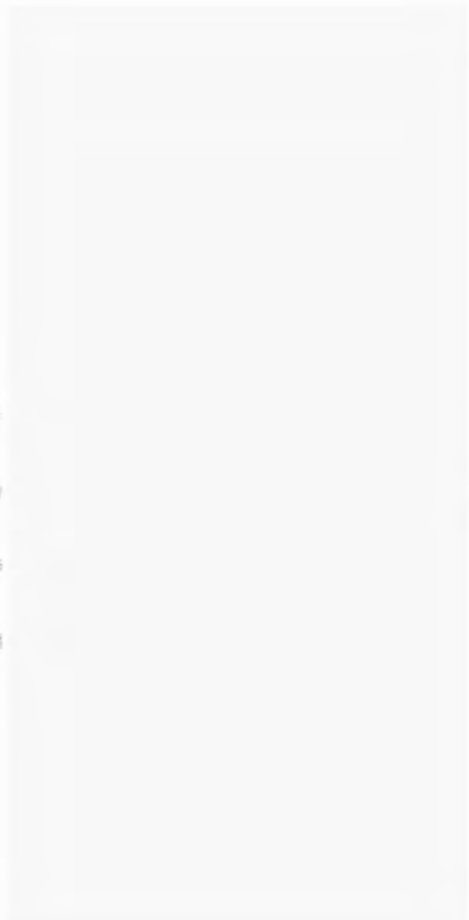
X 120,000.

PLATE 30.

A photograph of a plasmodesma
communicating between a "hinge
cell" and a central cell of the
sensory hinge.

X 120,000.

30
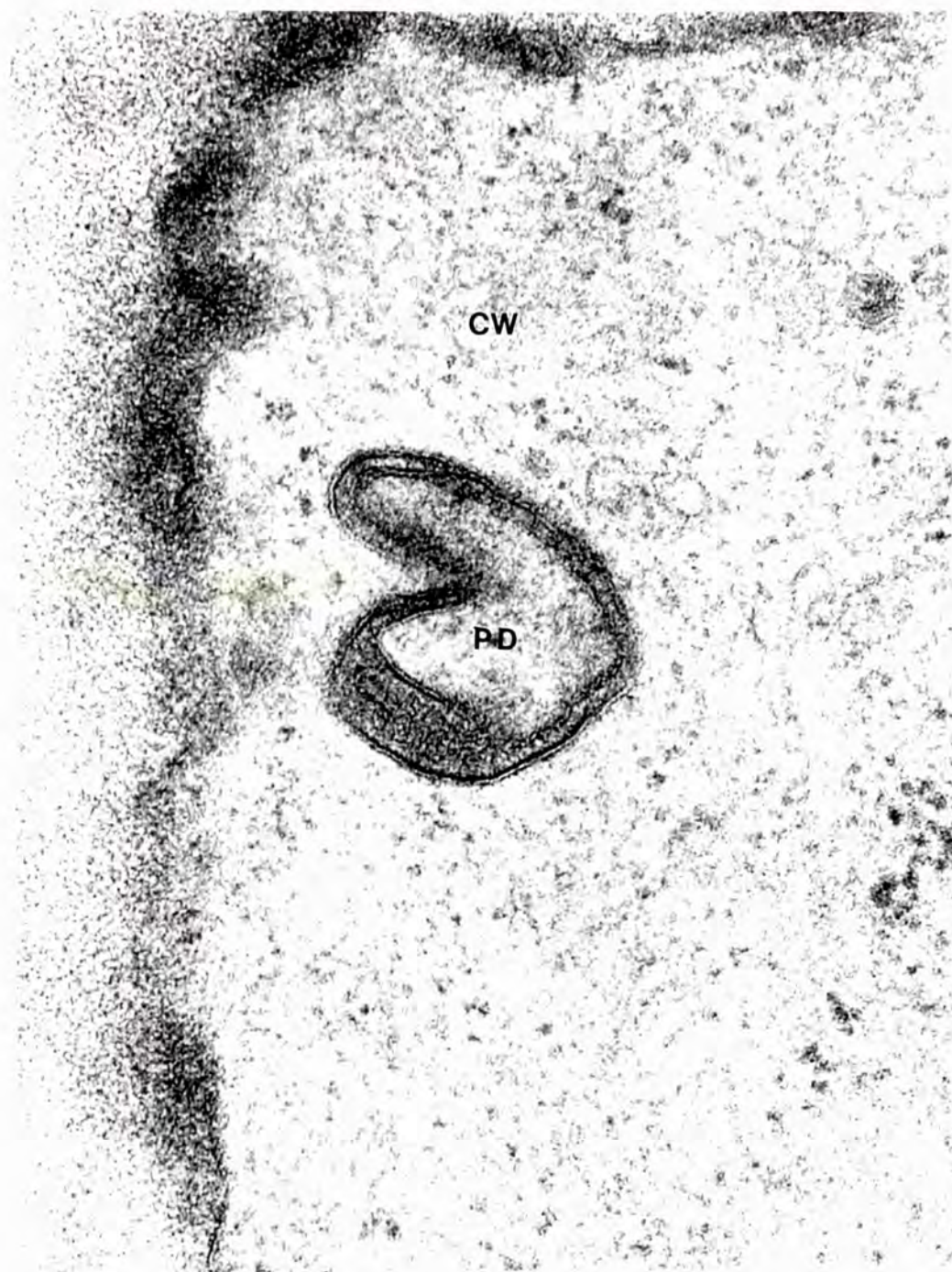
PLATE XI

The top of a cell of the sensory hair hinge
showing the typical prochastid type of
organelle, and the cell above, which has
a usual chlorcine. The plasmodesmata
are also shown.

X 20,000.

PLATE 31

The top of a cell of the sensory hair hinge
showing the typical proplastid type of
organelle, and the cell above, which has
a usual chloroplast. The plasmodesmata
are also shown.
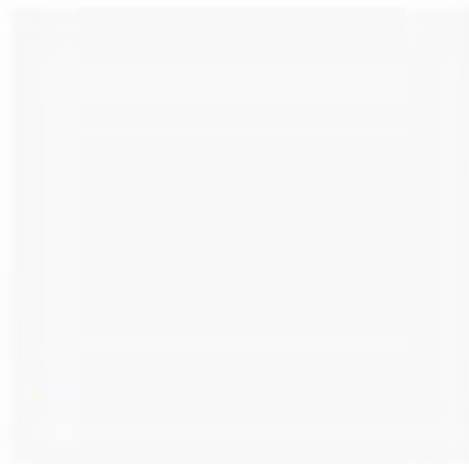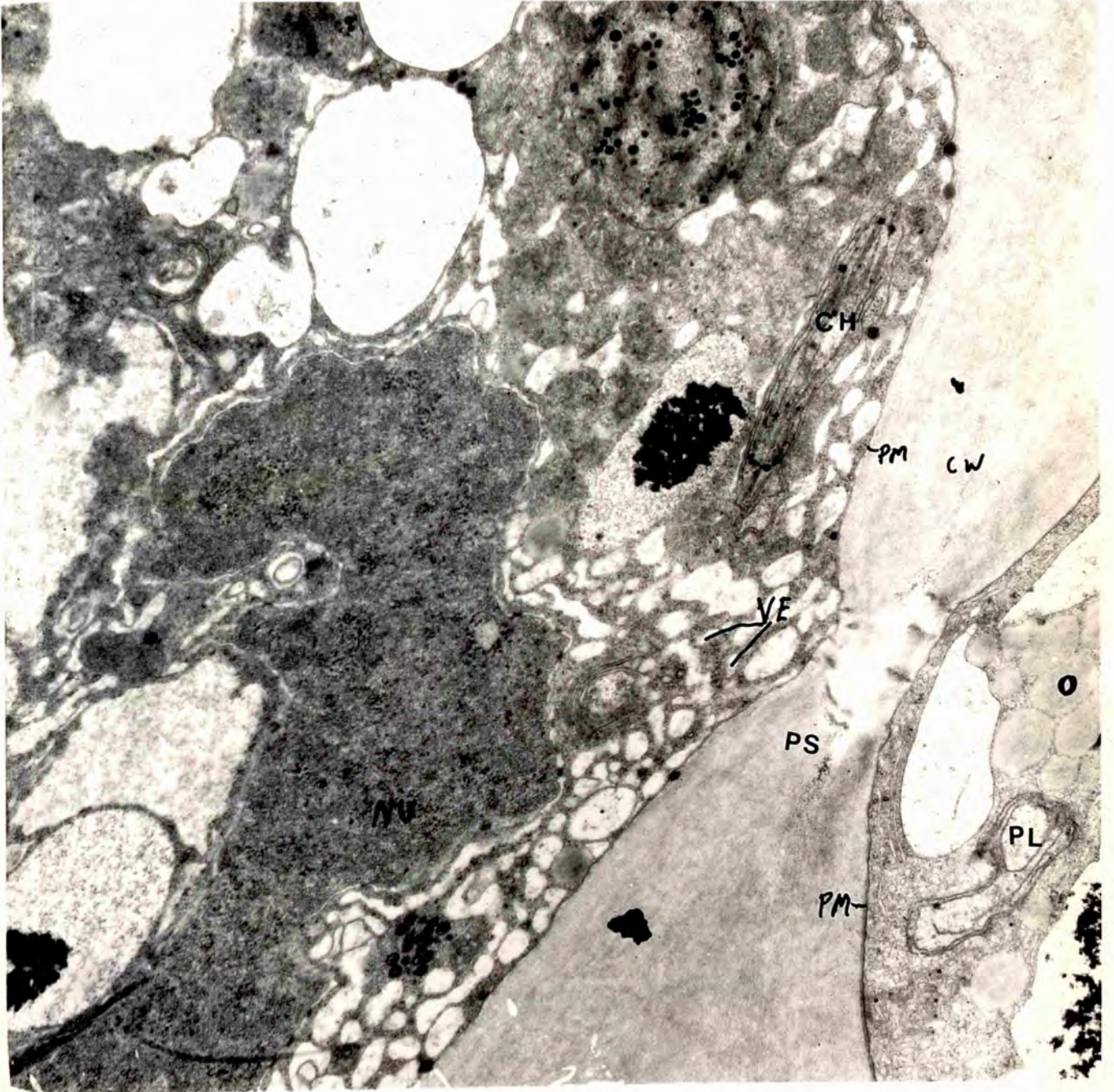
X 20,000.

31

PLATE 32

Portion of a sensory hinge cell shown
in plate 25, but at a higher
magnification. This photograph
illustrates the numerous vesicles
which occur.

X 40,000.

PLATE 32

Portion of a sensory hinge cell shown

in plate 23, but at a higher

magnification. This photograph

illustrates the numerous vesicles

which occur.

X 40,000.

32
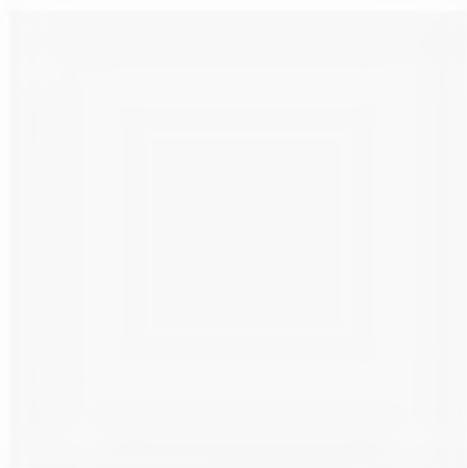
PLATE 33.

High power photograph of the cell
wall dividing the hinge cell from the
"conducting cell", showing numerous
plasmodesmata . Portion of the section
shown in plate 23.

X 120,000·

33

PLATE 34.

High Power photograph of section shown

in plate 23, showing transverse sections

of plasmodesmata which occur in the

thick cell wall at the hinge.

X 170,000.

PLATE 34.

High Power photograph of section shown

in plate 23, showing transverse sections

of plasmodesmata which occur in the

thick cell wall at the hinge.

X 170,000.

34
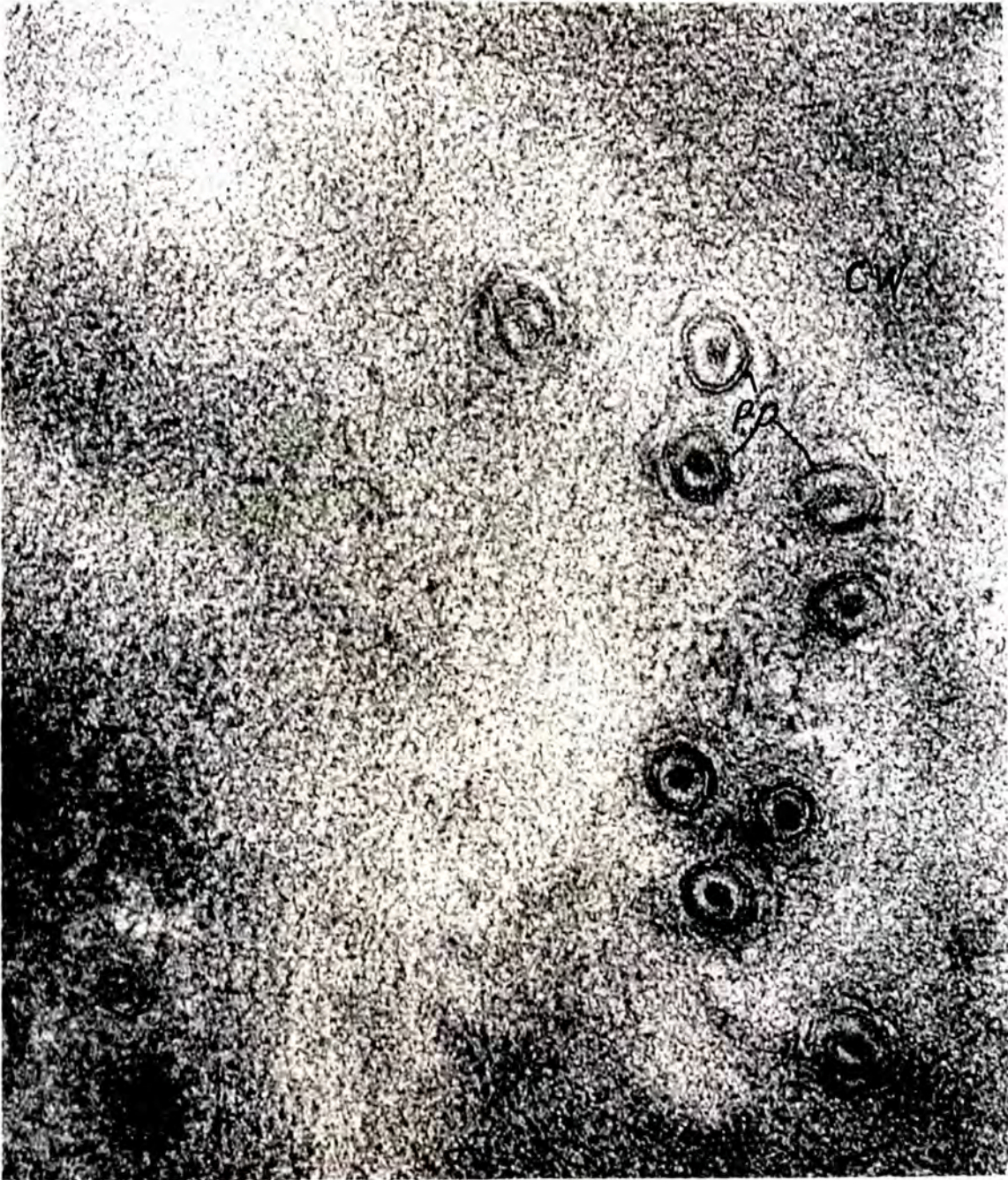
Plate 35

in junction of the lever portion of
the memory hair with the cells above
it us. The cells of the lever region
consist of long fibres.

X 15000.

PLATE 35

The junction of the lever portion of
the sensory hair with the cells above
hinge. The cells of the lever region
consist of long fibres.

X 15000.

35

Plate 26

ction of sensory hinge cell from
the same type as represented in
plate 23, but from a different hinge
cell and section. In this photograph
the endoplasmic reticulum appears to
be breaking up into vesicles.
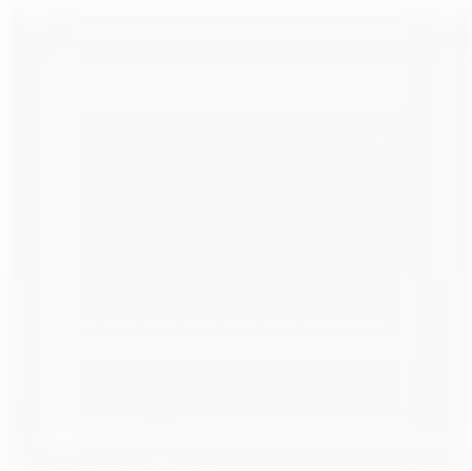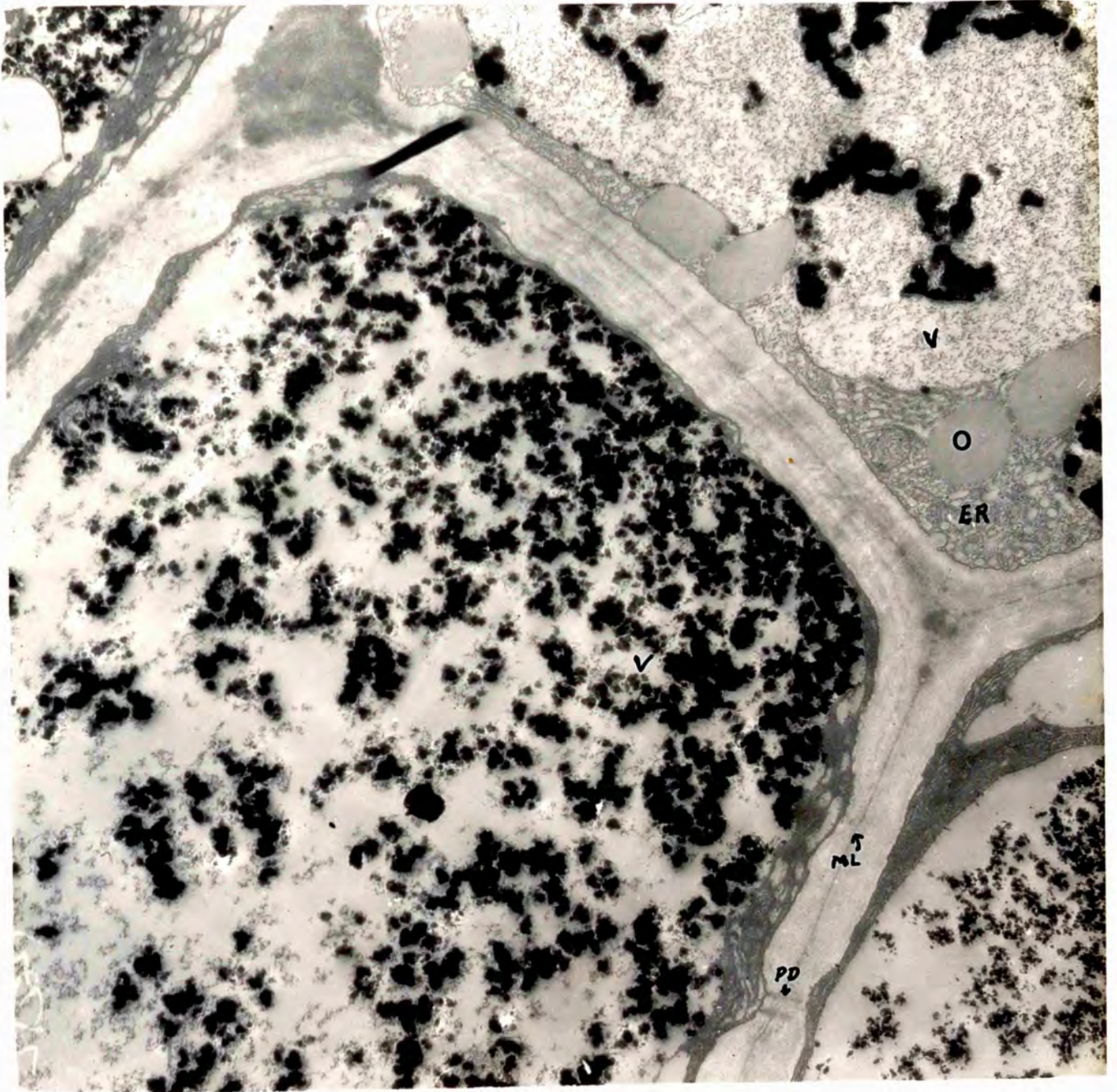
X 12000.

PLATE 36

Portion of sensory hinge cell from
the same hinge as represented in
plate 23, but from a different hinge
cell and section. In this photograph
the endoplasmic reticulum appears to
be breaking up into vesicles.

X 12000.

36
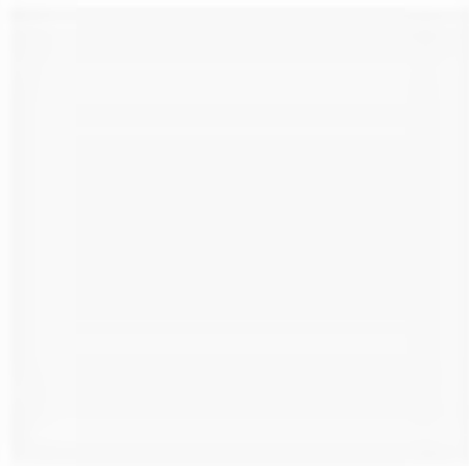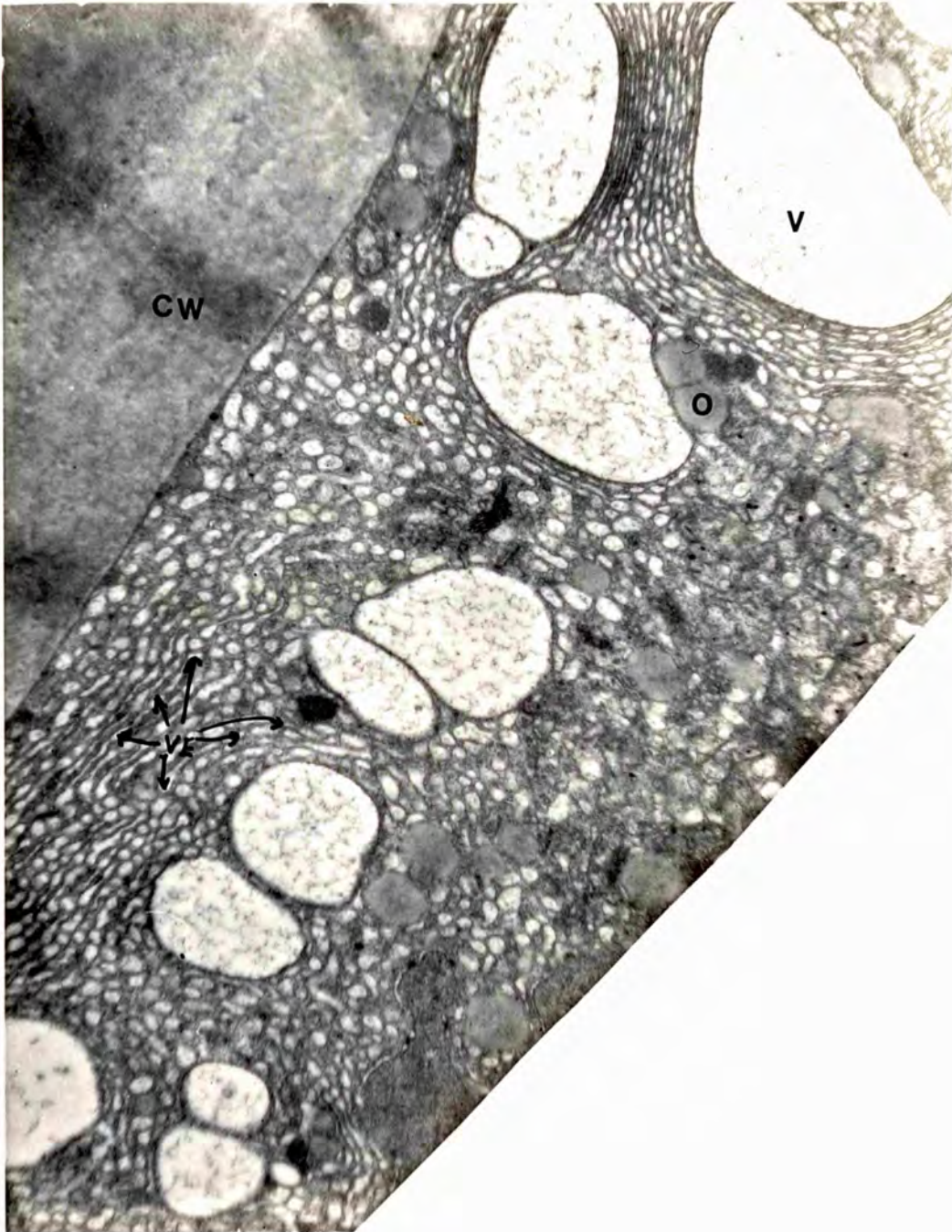
PLATE 37, 38.

Portions of the same sensory hinge,

in the same series of sections as

shown in plate 23, but an adjacent

hinge cell. Note again the

clustering of organelles near the

centre, and the appearance of the

cuticle.

X 15000

37
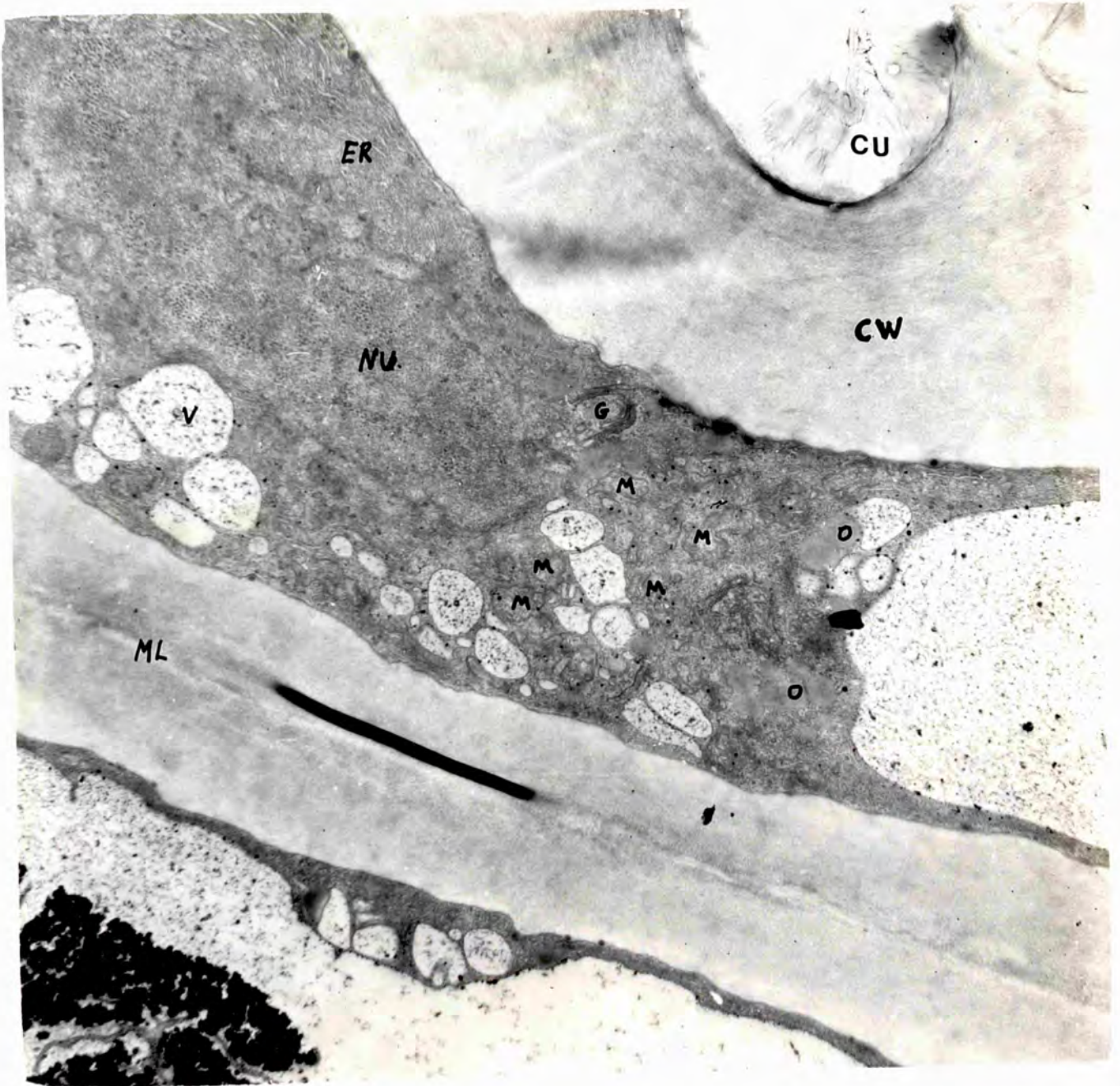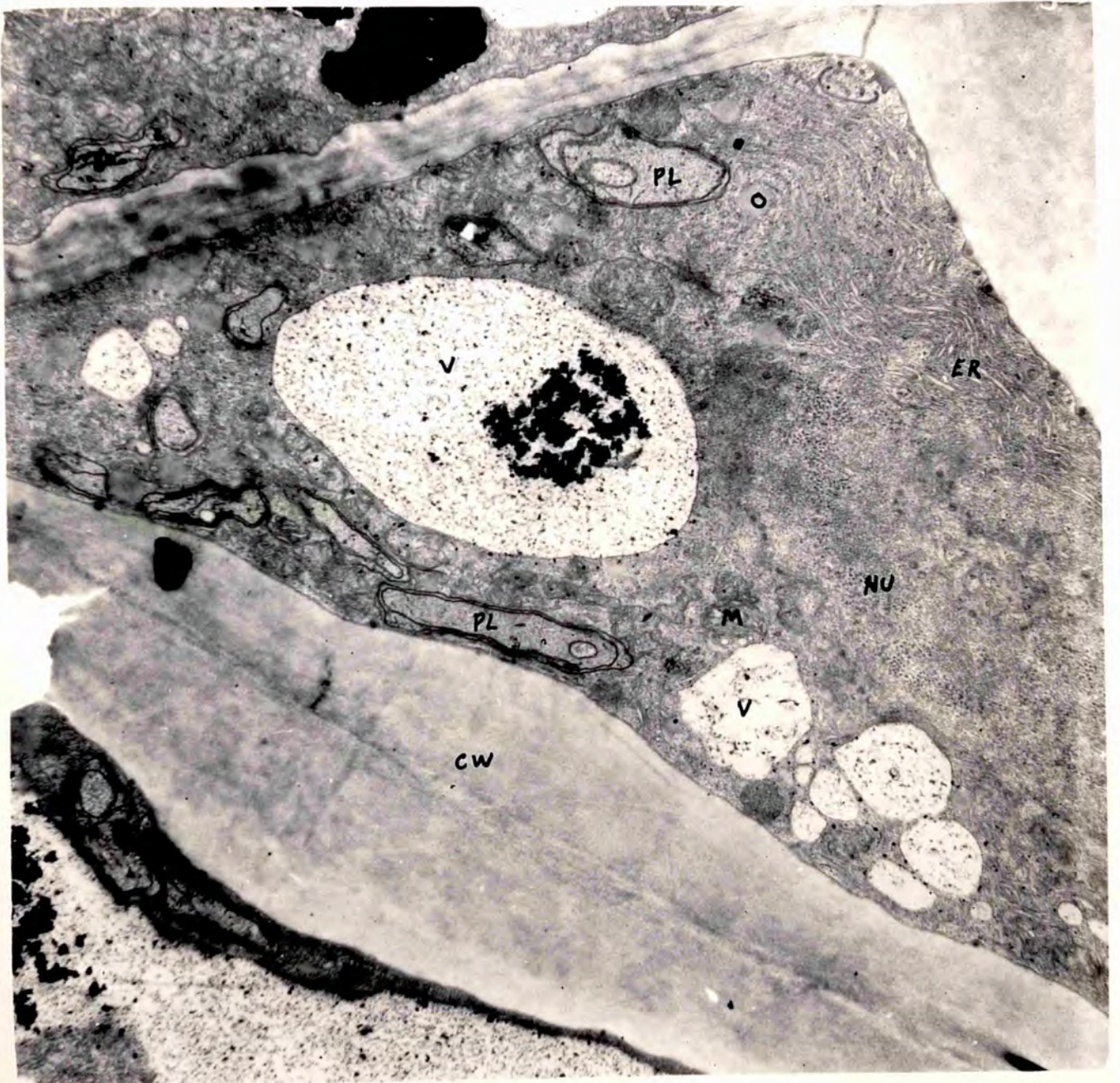
PLATE 39

In these central cells of the sensory
hair hinge, pitting is seen where
plasmodesmata pass through. This is
not a general phenomenon.

Fixation was with glutaraldehyde,
post-fixation was with osmic acid,
staining was done using uranyl acetate
and lead citrate.

X 10,000
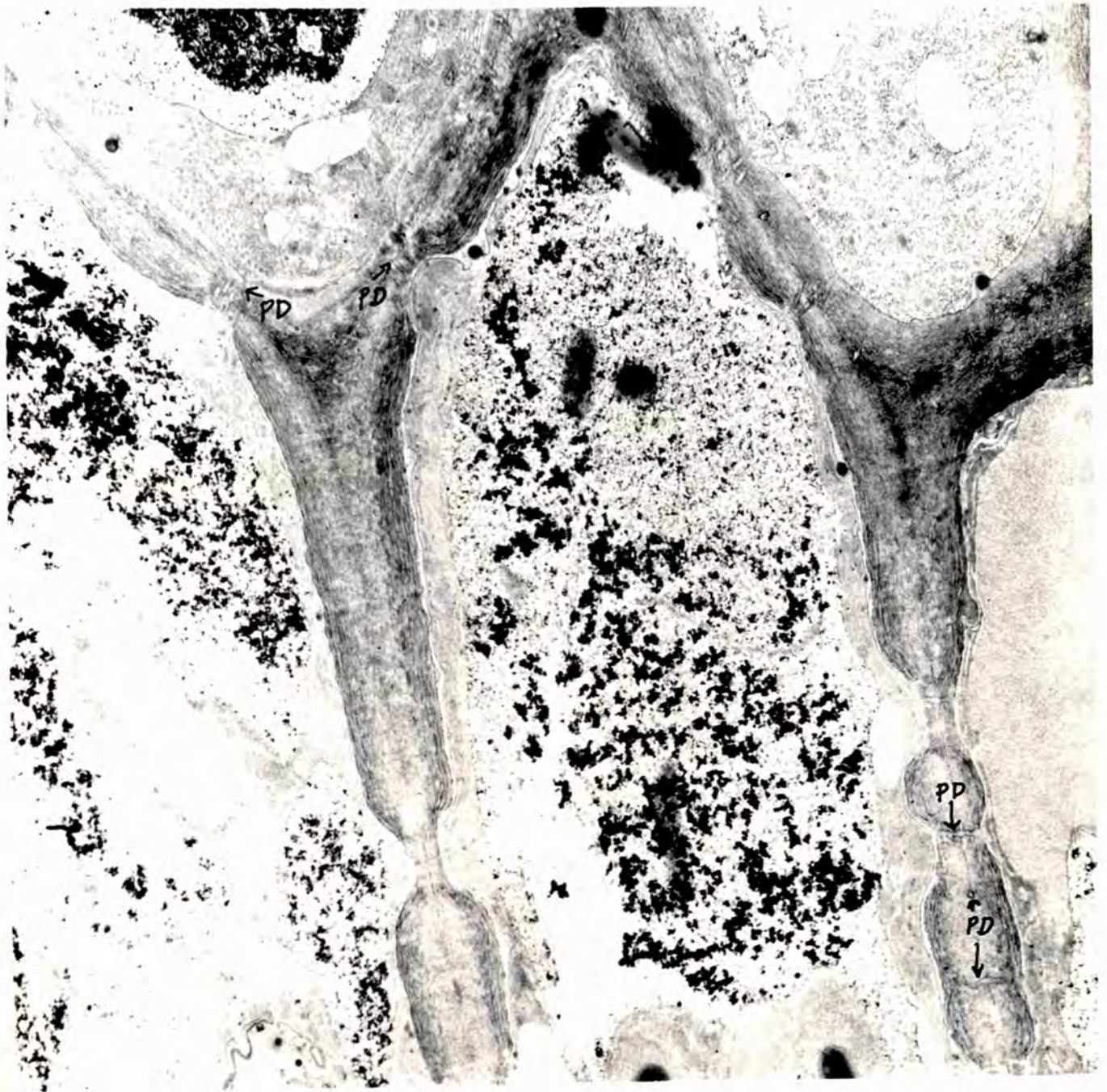
39

PLATE AO.

In this _encapsulated_ sensory hinge
cell shown in the drawing of plate
14, fixation with glutaraldehyde was
extended from three to six hours,
giving a more intense staining and
contrast in the final result.
Post-fixation with osmic acid,
stained with uranyl acetate and lead
citrate.

PLATE 40.

In this <u>unstimulated</u> sensory hinge
cell shown in the drawing of plate
14, fixation with glutaraldehyde was
extended from three to six hours,
giving a more intense staining and
contrast in the final result.
Post-fixation with osmic acid,
stained with uranyl acetate and lead
citrate.

40
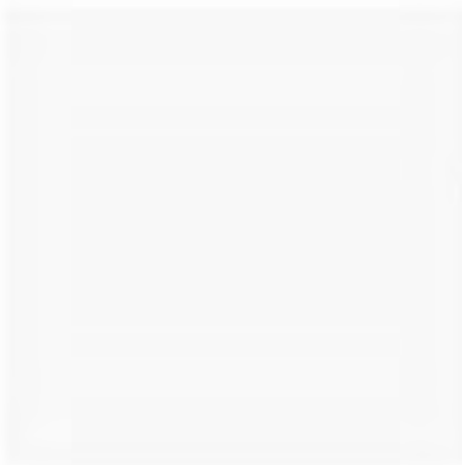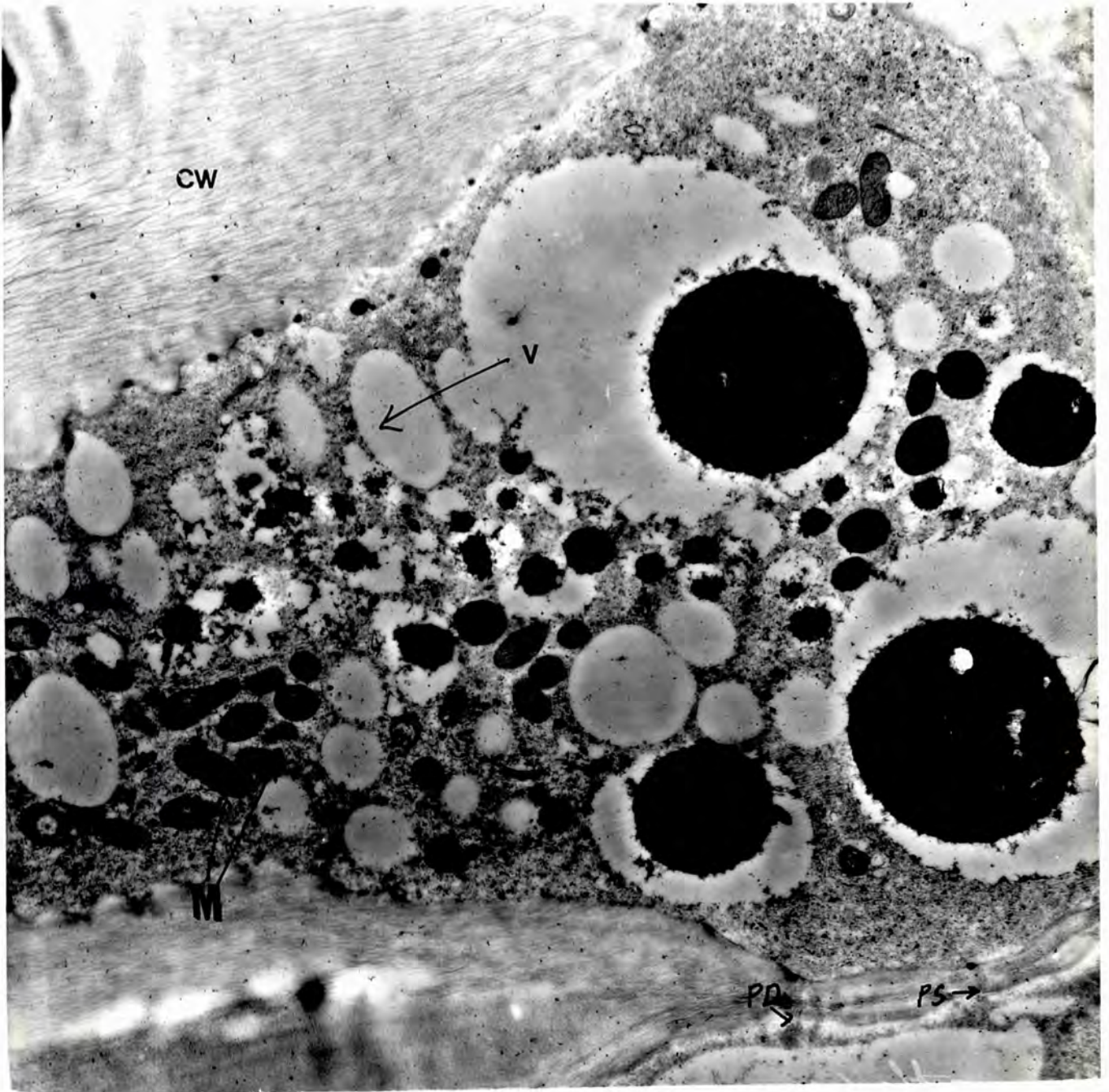
PLATE AI.

Low power photograph of the sensory
hairs in the stimulated, bent position.
This is represented in the drawing
shown in plate 17. Fixed in
glutaraldehyde, post-fixed in osmic
acid. The intense staining of the
cuticle and middle lamella after
bending the hair, is noteworthy.

X 2000

PLATE 41.

Low power photograph of the sensory

hinge in the stimulated, bent position.

This is represented in the drawing

shown in plate 17. Fixed in

glutaraldehyde, post-fixed in osmic

acid. The intense staining of the

cuticle and middle lamella after

bending the hair, is noteworthy.

X 2000

PLATE A2
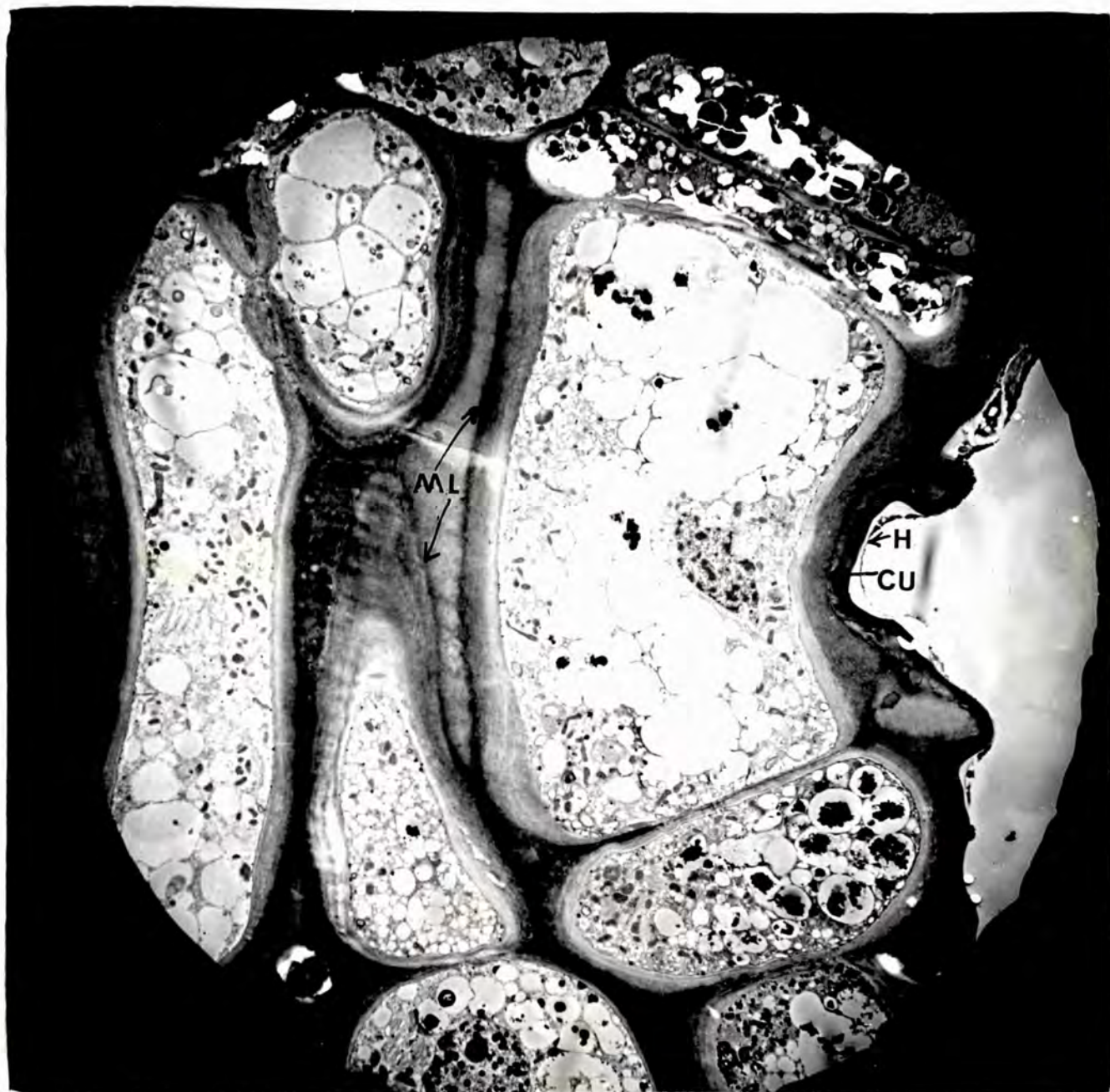
A photograph of portion of plate A1.
The above the plasmolemma, and
the alterations which occur in the
appearance of membrane and
organelles after stimulation.

X 8000

PLATE 42

A photograph of portion of plate 41.
The shows the plasmodesmata, and
the alterations which occur in the
appearance of membranes and
organelles after stimulation.

X 8000

PLATE A3
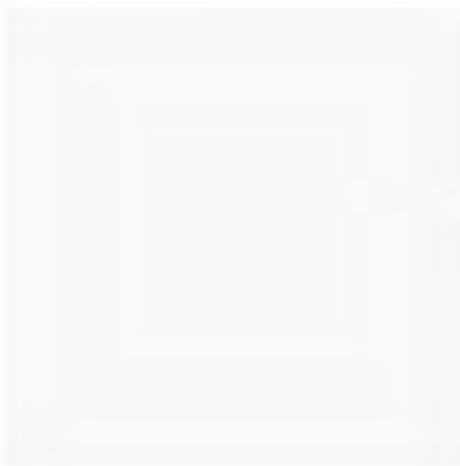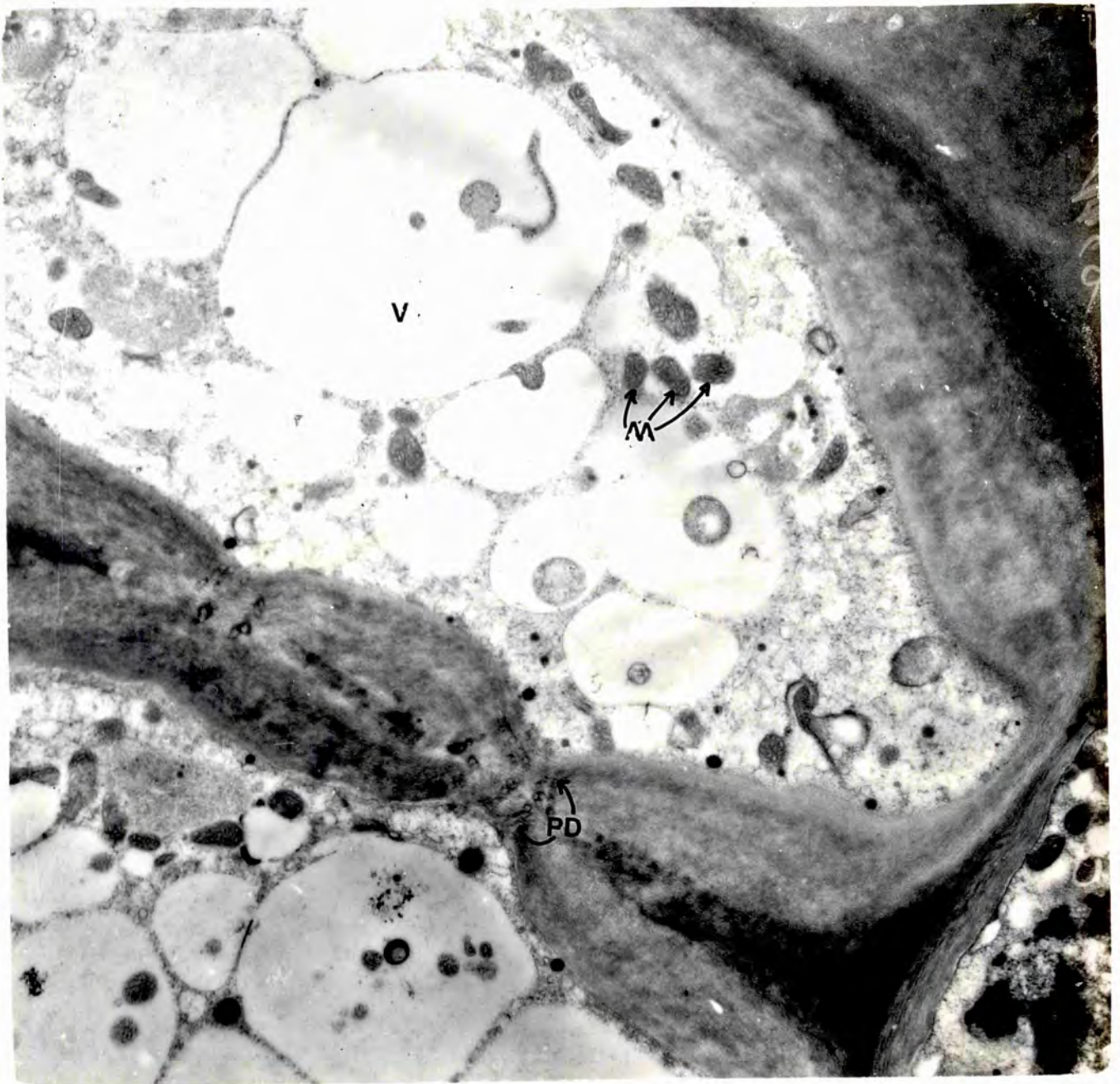
A photograph of portion of plate A1.
This shown the alteration in the
appearance of membrane and
organelles after bending.

X 8000

PLATE 43

A photograph of portion of plate 41.

This shown the alteration in the

appearance of membranes and

organelles after bending.

X 8000

43
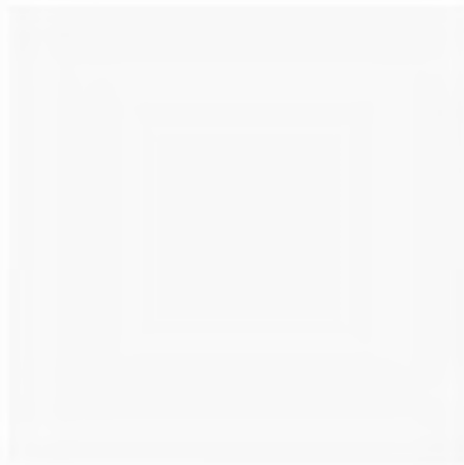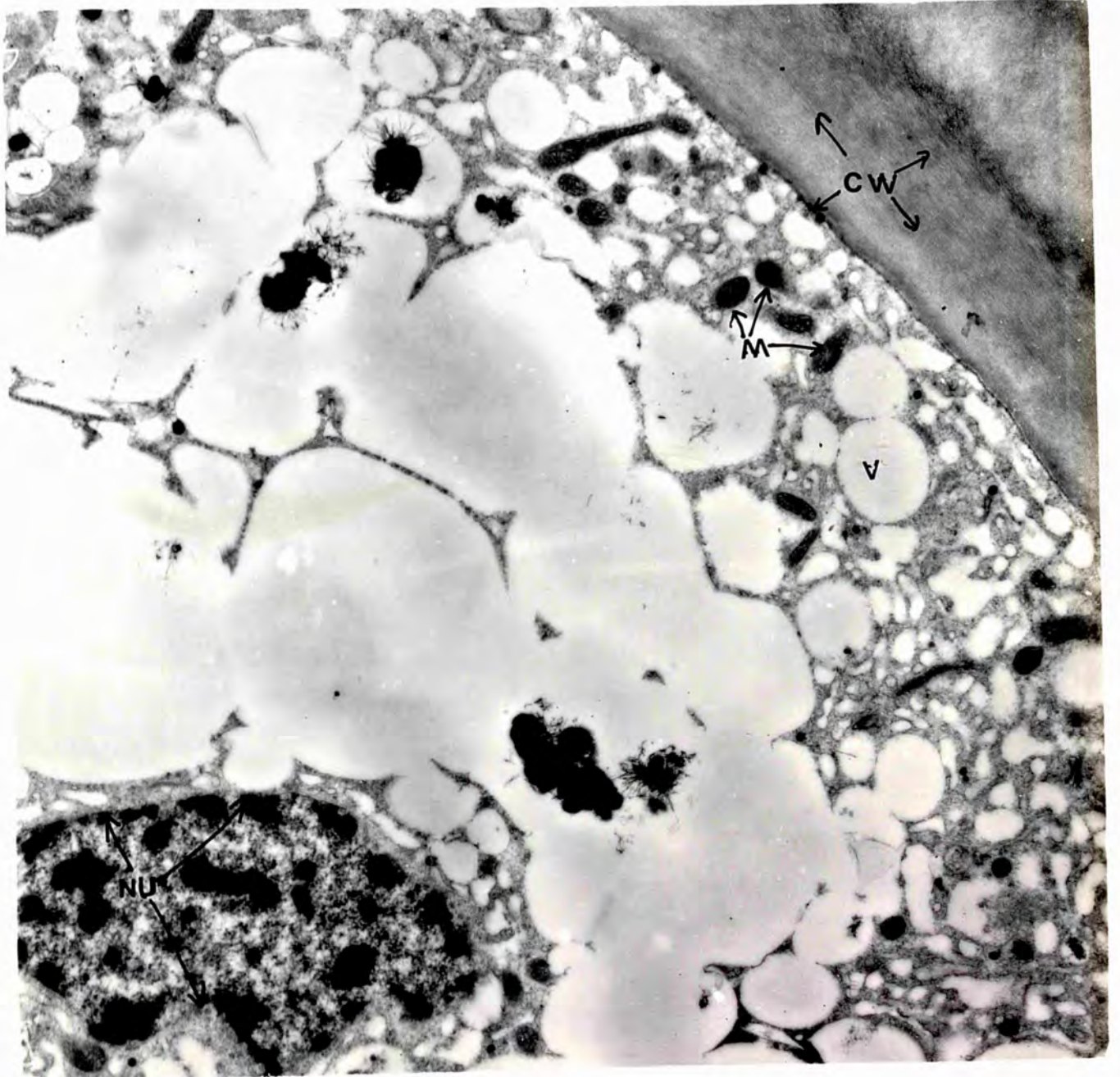
PLATE 44.

A low power photograph of the
sensory hinge adjacent to
plate 41, and represented in the
drawing shown in plate 17.

X 2000
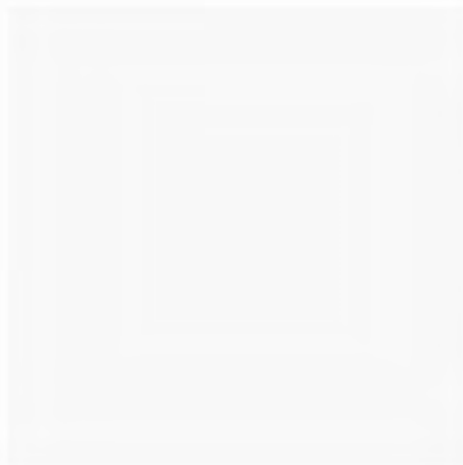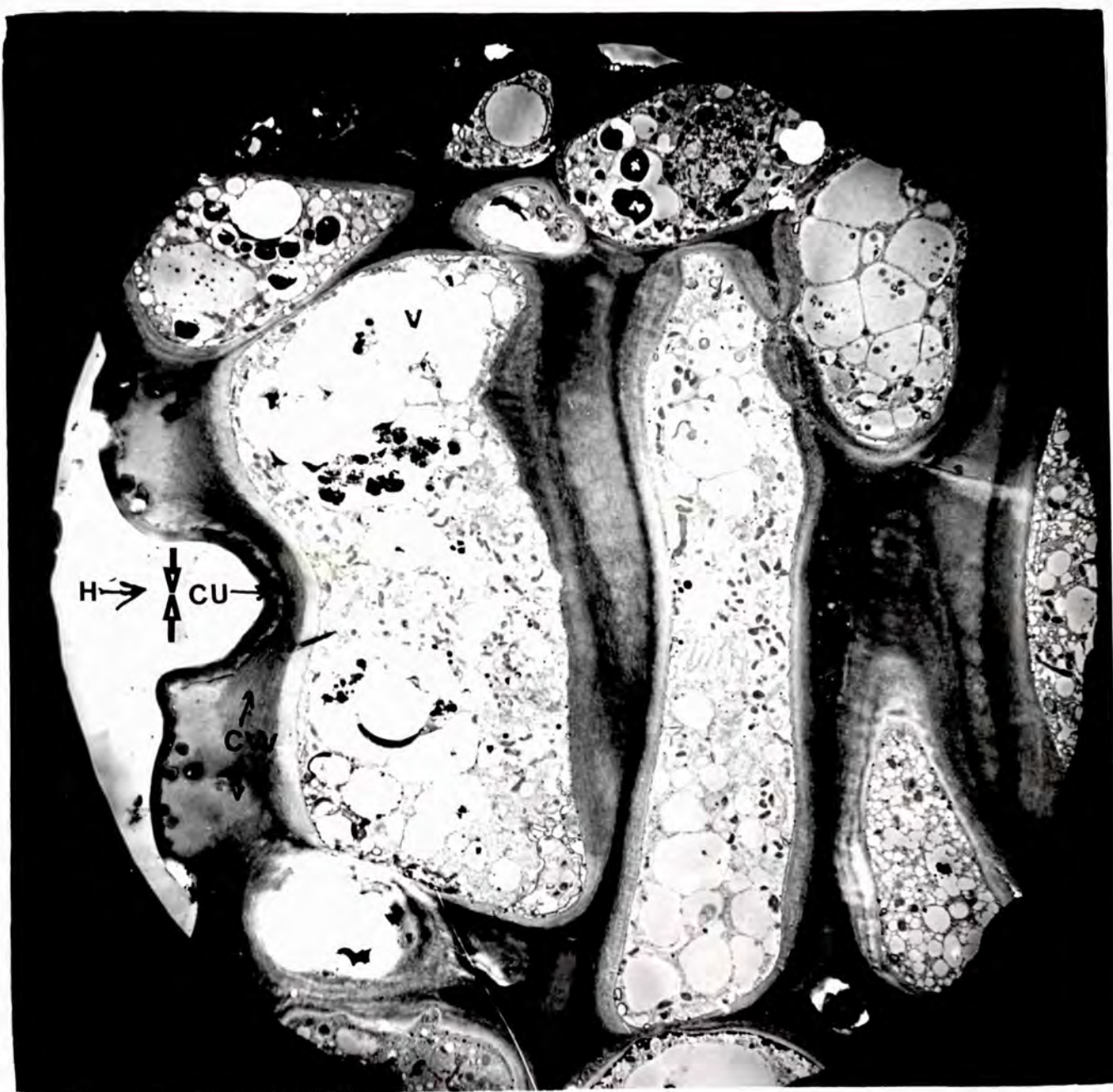
44

PLATE 45.

A high power photograph of the sensory

hinge after bending, shown in plate 44.

The middle lamella has a fibrous

structure, and a type of intense

staining not shown in the unstimulated

hair.

X 20,000.

PLATE 45.

A high power photograph of the sensory
hinge after bending, shown in plate 44.
The middle lamella has a fibrous
structure, and a type of intense
staining not shown in the unstimulated
hair.

X 20,000.

45
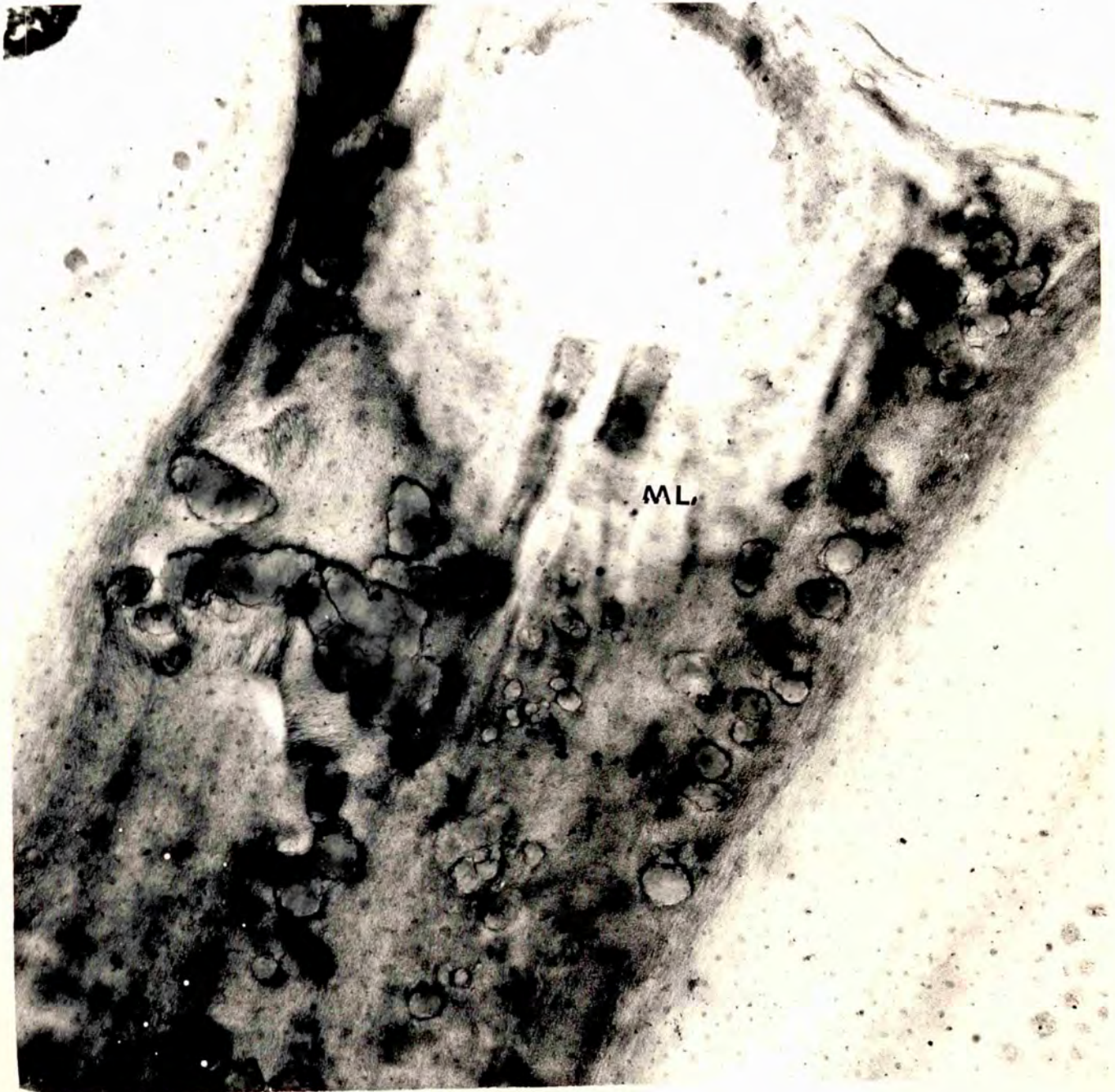
PLATE 46

A photograph of portion of hinge cell

of the sensory hair stimulated, and fixed

in the bent position. This section came

from the same series as illustrated in

plate 18, and shows the appearance of

membranes surrounding the nucleus,

mitochondria, plastids, vacuoles and the

plasmalemma.

Fixation was with glutaraldehyde,

post-fixation was done with osmic acid,

staining was done with uranyl acetate and

lead citrate.

X 40,000.

PLATE 47
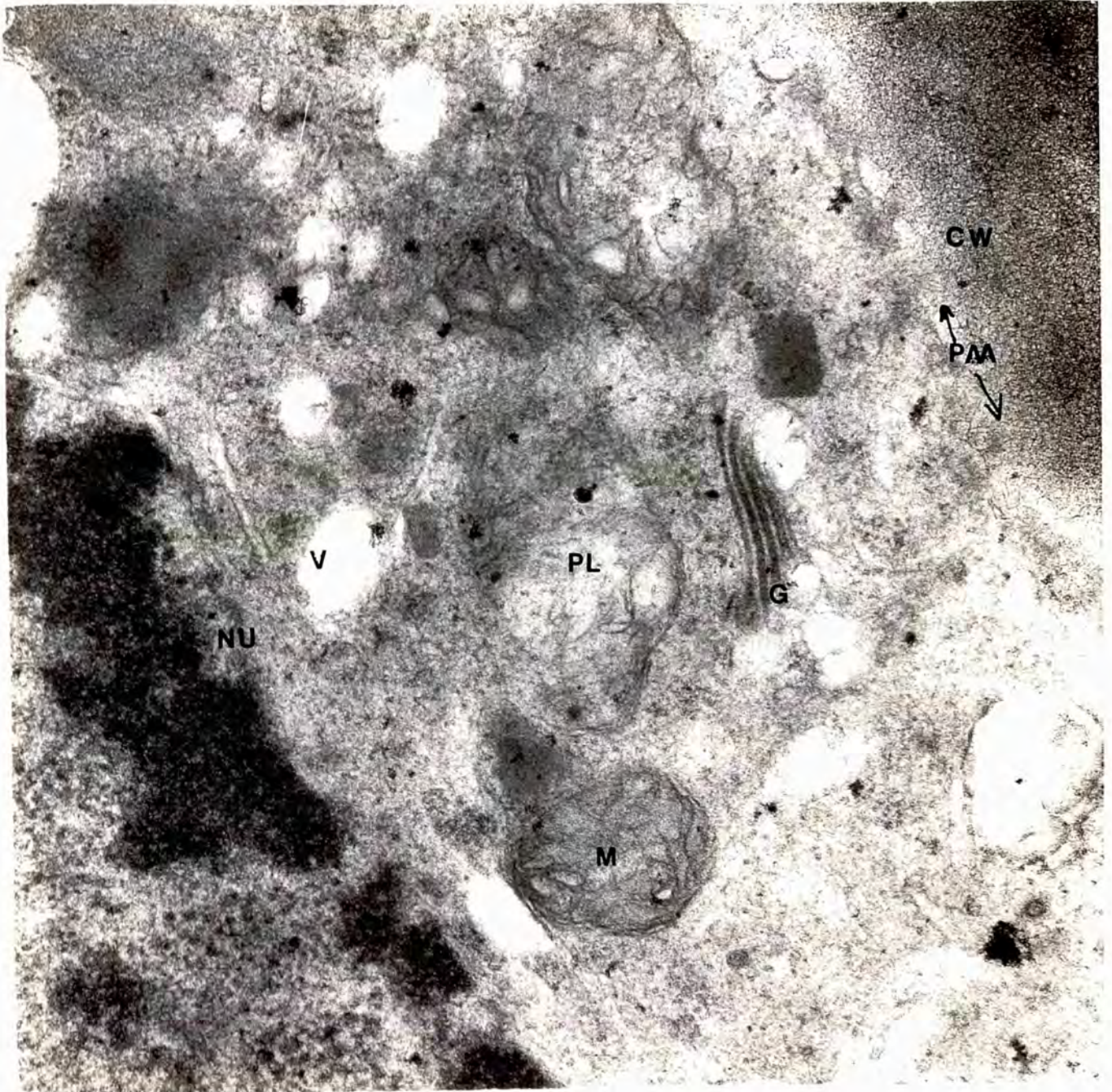
Portion of the sensory hinge after bending,
and represented in the drawing on plate 19.
Differences in the membranes, a deep
staining of the cell walls, and swollen
organelles are noteworthy. Glutaraldehyde
fixation, osmic acid post-fixation, uranyl
acetate-lead citrate staining.

X 10,000.
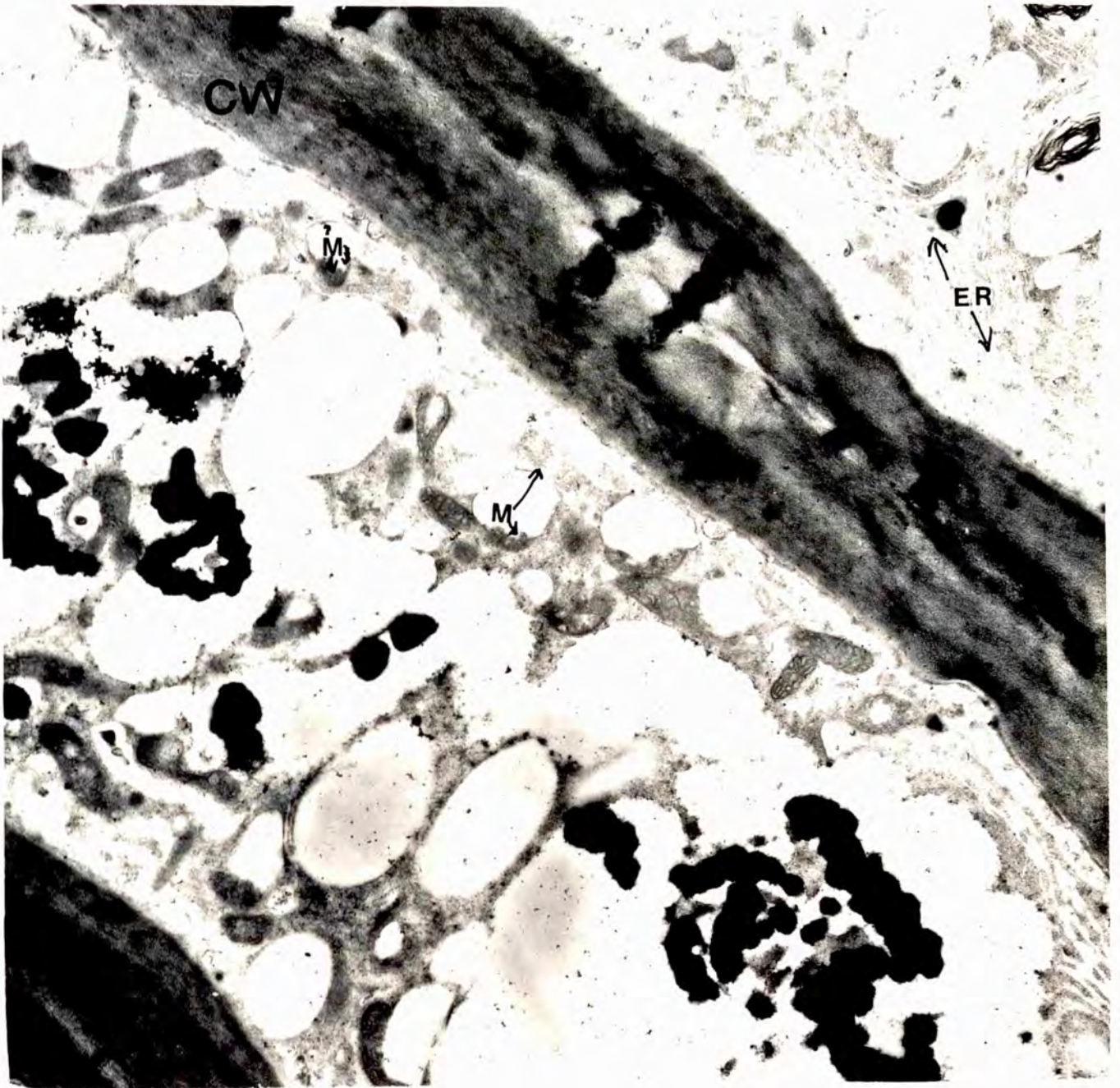
his is another portion of the same
section shown in plate 17, and represented
in the drawing on plate 15. Some
chromoplasts have swollen greatly, and
cells of membranous material is present
in some vacuoles.

X 10,000.

PLATE 48

This is another portion of the same
section shown in plate 47, and represented
in the drawing on plate 19. Some
mitochondria have swollen greatly, and
coils of membranous material is present
in some vacuoles.
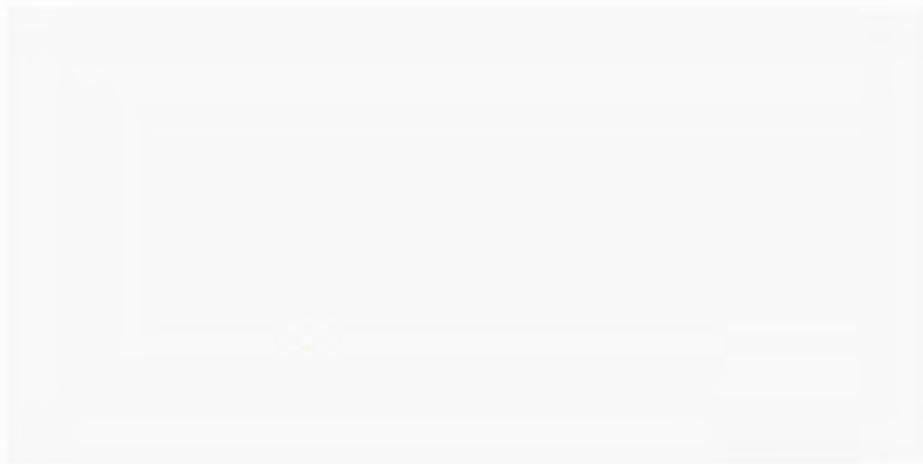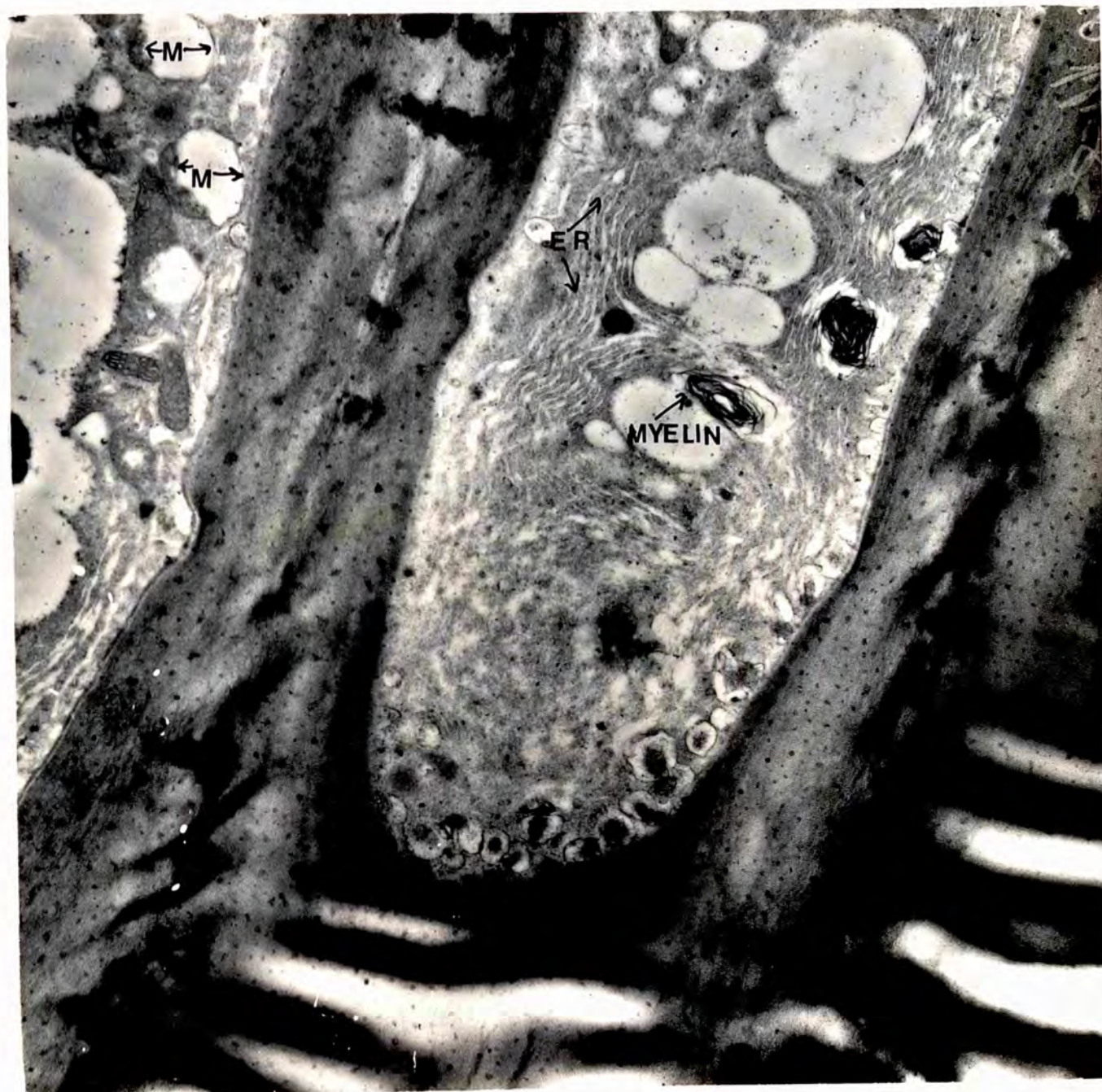
X 10,000.

PLATE 48

This is another portion of the same section shown in plates 19, 47 and 48. Note the unusual vacuoles with internal deposits, the swollen mitochondria, and the appearance of membranes.

X 10,000.

PLATE 49

This is another portion of the same section
shown in plates 19, 47 and 48. Note the unusual
vacuoles with internal deposits, the swollen
mitochondria, and the appearance of membranes.

X 10,000.

PLATE 50

— i i —
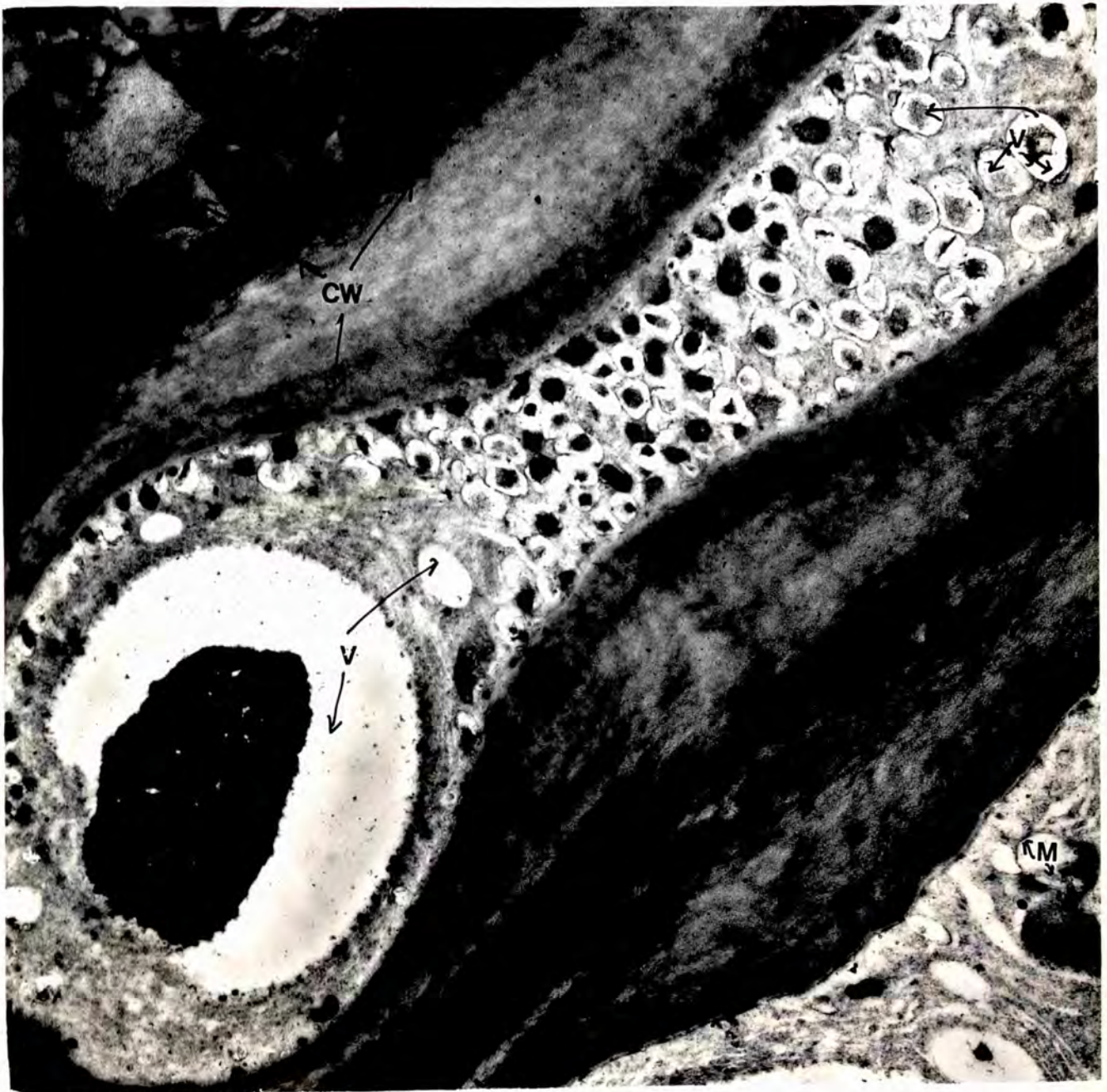
A sensory hinge cell fixed in the
bent position, and represented in
the drawing shown in Plate 20. This
section shows especially the alteration
in the cell walls with bending.

Stimulated.

Fixed in glutaraldehyde, post-fixed
with osmic acid; stained with uranyl
acetate- lead citrate.

X 10,000
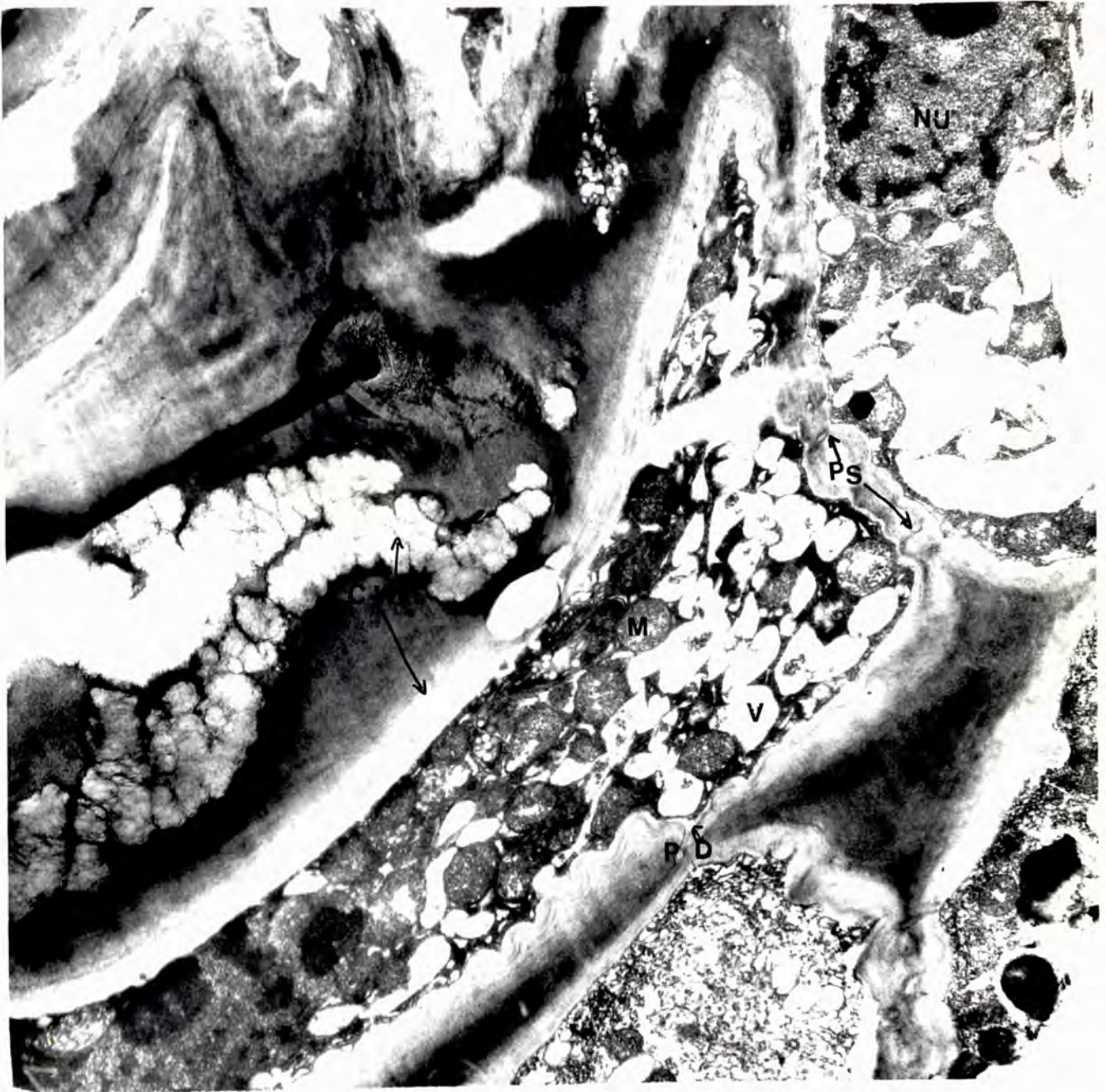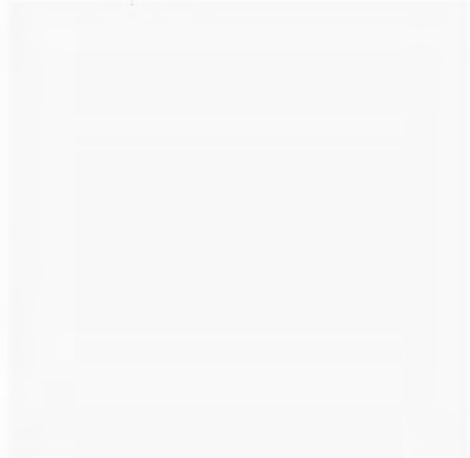
142

PLATE 51.

simulated, sensory image cell in the head position; this is portion of the same cell shown in plate 50, and in the drawing of plate 2. The purpose of this photograph is to illustrate the structure of the cell walls after bending.

X10,000.

PLATE 51.

A <u>stimulated</u>, sensory hinge cell in the bent position; this is portion of the same cell shown in plate 50, and in the drawing of plate 20. The purpose of this photograph is to illustrate the structure of the cell walls after bending.
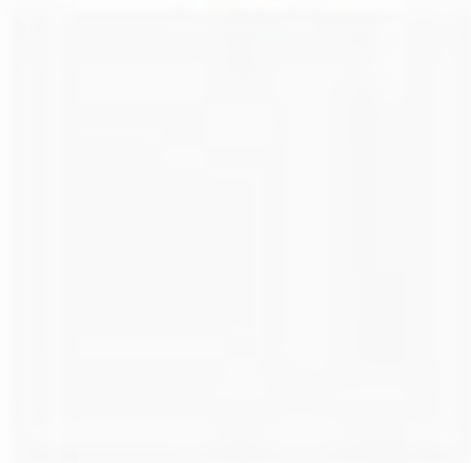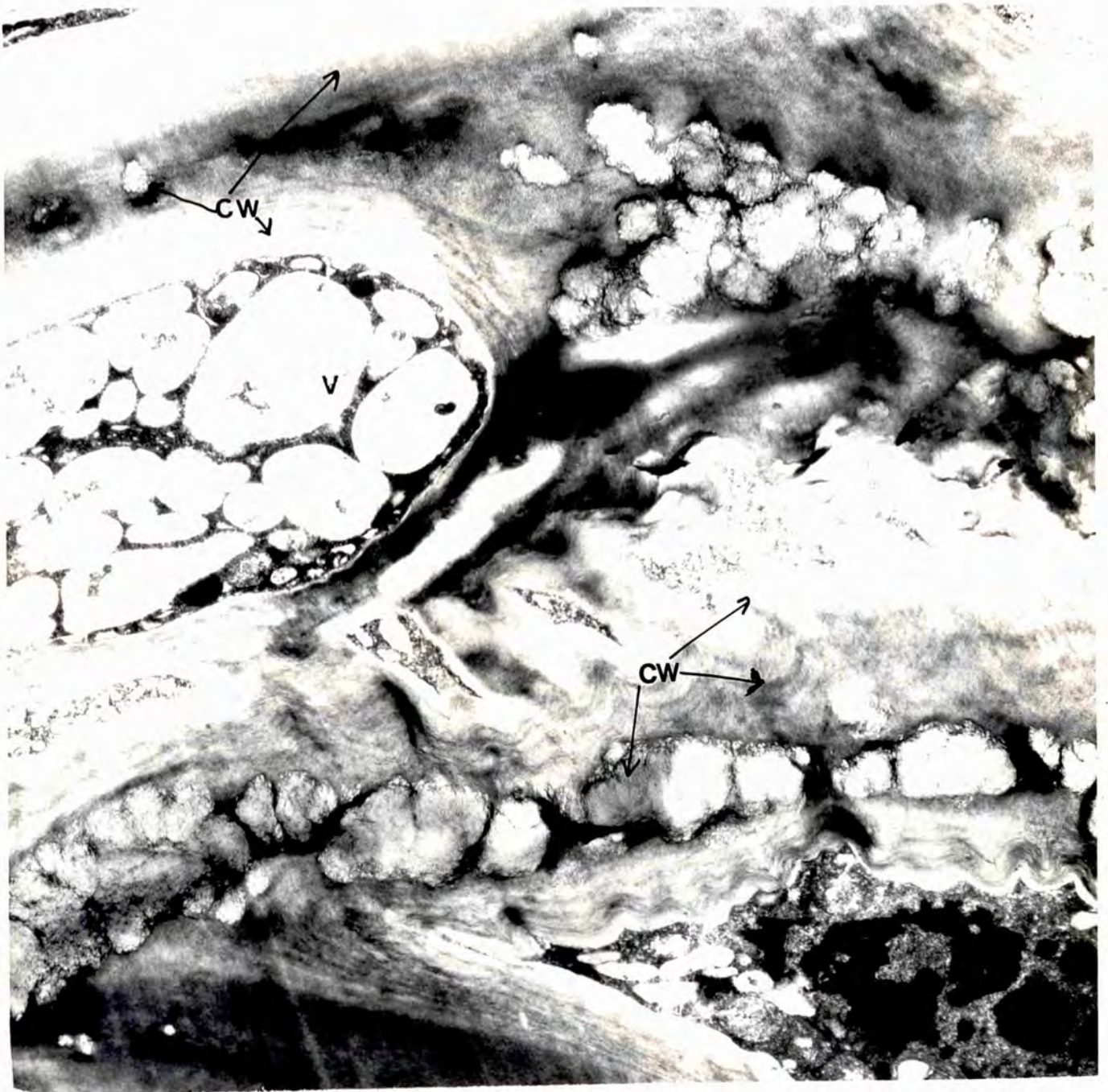
X10,000.

The stimulated hive, and the cells
below it. This photograph presents
the bottom right hand section of
plates A1 and 17, taken at a higher
magnification.

X 10,000.

PLATE 52

The stimulated hinge, and the cells
below it.   This photograph presents
the bottom right hand section of
plates 41 and 17, taken at a higher
magnification.
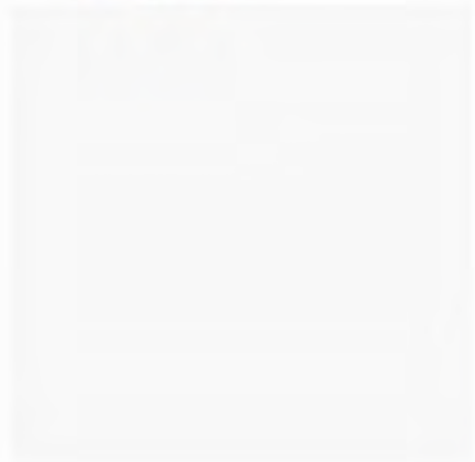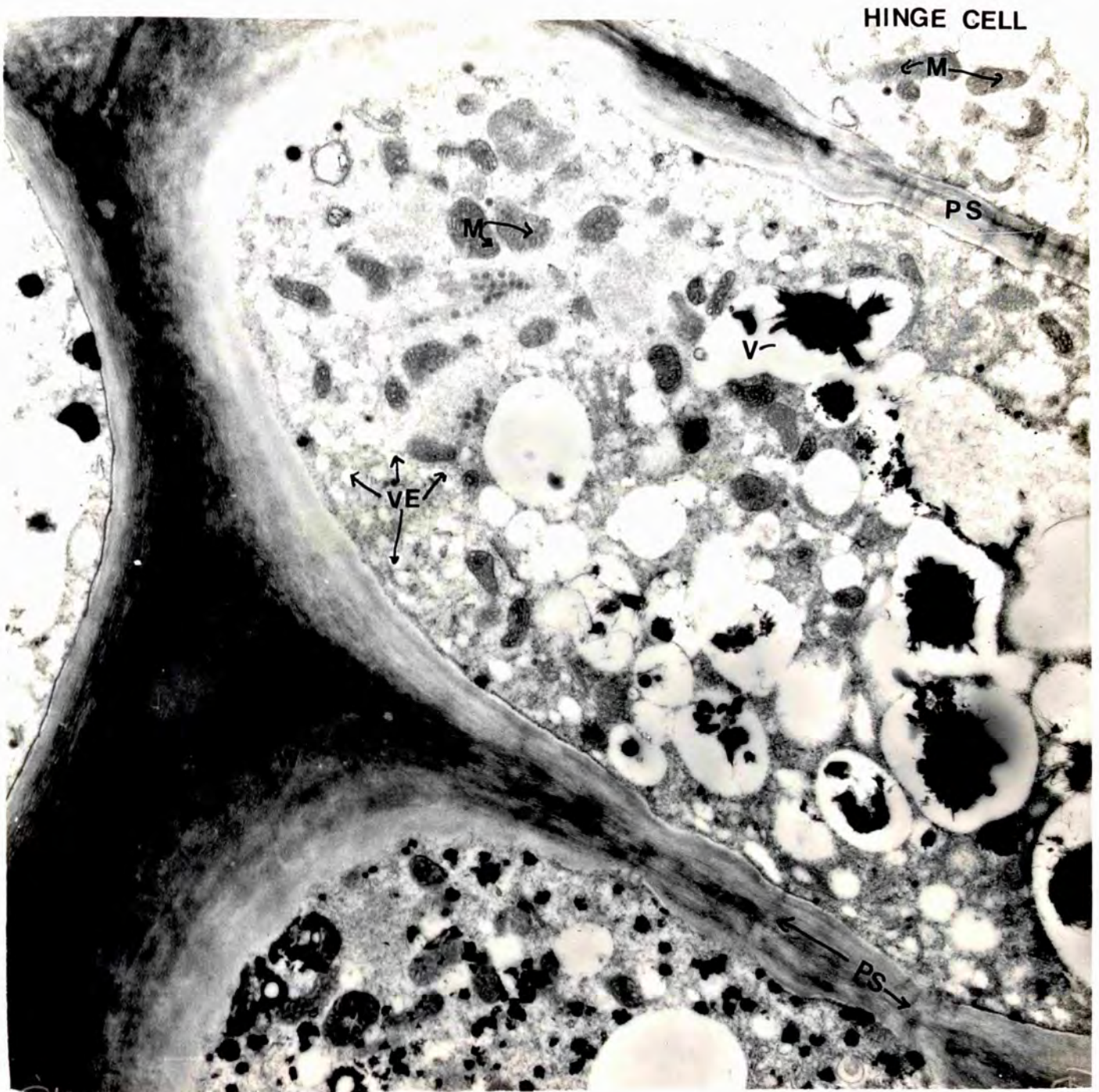
X 10,000.

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PLATE 52

Golgi apparatus in sensory

hair hinge.

Permanganate fixed.

X 60,000.

PLATE 53

Golgi apparatus in sensory

hair hinge.

Permanganate fixed.

X 60,000.
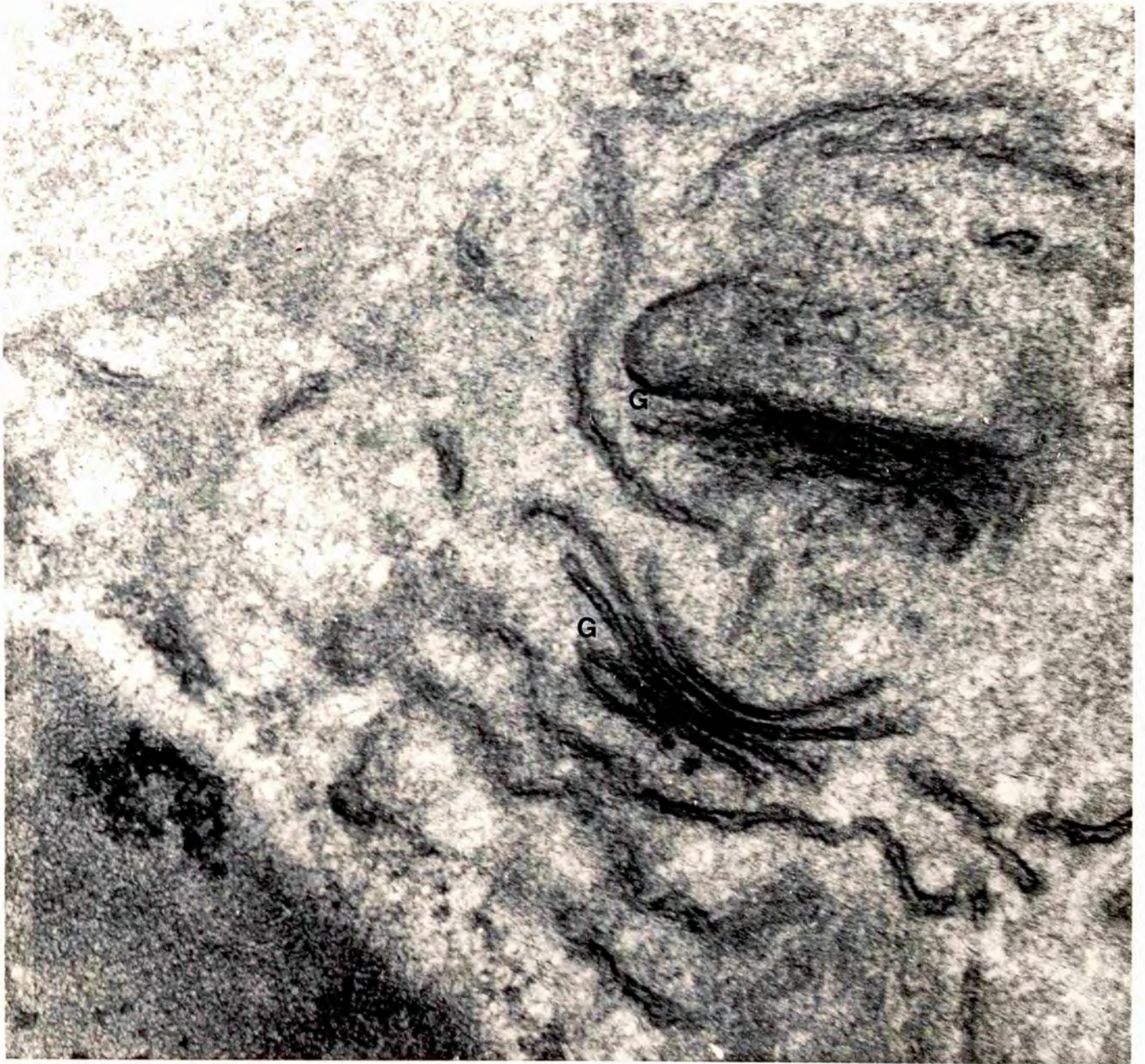
(2. GENERAL DESCRIPTION OF THE FINE STRUCTURE, continued.)

Features of the typical hinge are the thick cuticle, usually at least 1 micron; the massive cell walls which above and below the furrow of the hinge cells may exceed 10 microns in thickness, the large numbers of mitochondria clustered along the transverse plane of the hinge, and typically 1 micron in diameter; the large nucleus which is lobed downwards towards the lamina, with a single nucleolus; very large vacuoles; some proplastid bodies usually about 3 x 5 microns in area; numerous vesicles; and abundant ER.

Reference to sections from a number of sensory hairs fails to reveal any apparent difference between the "hinge" cells or the "conducting" cells in fine structure. The numerous plasmodesmata opening downwards to the podium, and thence to the lamina of the trap from both hinge and conducting cells, makes it unnecessary to consider that the function of the conducting cells is different from that of the hinge cells.

Vesicles appear to arise in two ways: 1) by the breaking up of the ER, as appears to be happening in Plate **3B**; 2) by budding off from the Golgi apparatus, as shown in Plate 2**B**.

The ER and vesicles are almost exclusively smooth, as shown in Plates 25, 26, 27, 28 and 32, but an abundance of ribosomes and polysomes does occur (e.g. Plate 29). No case could be found where the ER opened into the plasma membrane.

In unstimulated, glutaraldehyde fixed material, the mitochondria

frequently showed an amoeboid appearance (Plate 26) but not so in permanganate fixed material (Plate 22). In stimulated tissue the mitochondria were often greatly swollen (Plate 47).

Both true chloroplasts, and the hinge cell plastid - as well as some mitochondria - contain densely staining granules, commonly referred to by numerous workers such as CHANCE (1963), as polyphosphate deposits.

Lipid droplets $\frac{1}{2}$ to 1 micron in diameter are common throughout the hinge cell cytoplasm, and usually appear as clear areas, as in Plate 26. Their identity was confirmed by staining hand cut sections with Sudan III stain, and by their complete absence from sections fixed with permanganate.

As shown in Plate 27, the vacuoles often contain precipitates, attributed by SCALA et al (1968 a), to the presence of large osmiophilic granules, which they termed "protein bodies," and compared them to aleurone grains. These may be seen in plates 23, 24, 25, 27, 28, 31, 32, 34, 35, 36, 37, 38 and 40.

An important feature of the hinge cells are the plasmodesmata. Only extremely rarely are these associated with pitting as shown in Plate 39. Plasmodesmata usually occur in groups, and connect the hinge to the conducting cells as shown in the upper left hand side of Plate 22, or the hinge with cells above or below, typified by the pore site in Plate 31. Small pores also pass through the outer hinge walls, and many plasmodesmata are extremely small, as

shown in Plate 30 where the pore is 0.3 µ in diameter, or Plate
33 where the diameter is about 0.25 µ, or Plate 34 where the
diameter is less than 0.08 µ.

Cells immediately above the hinge (Plate 31), and even as high
as the cells of the lever (Plate 35) and cells below the hinge
(Plate 41), and from other plates not presented, differ signifi-
cantly from the hinge cells in the type of plastid and the less
abundant ER. There is a big difference in the nature of the hinge
cells compared with the mesophyll of the trap as shown in Plate 10.
The main features of leaf trap mesophyll are the numerous chloro-
plasts 5 x 3 microns in size, the mitochondria, and limited ER,
but numerous vesicles.

The plastids of the sensory hinge were of a proplastid type, as
shown in plates 22, 26, 27, 28 and 38. These contain few lamellae,
and except in hairs which were fixed a week or more after reaching
maturity, very little starch, but the relative amount of starch
in the hinge plastid and in the true chloroplasts adjacent was
always comparable.

The walls of the hinge region in the unstimulated state merely
showed plasmodesmata, but fixed in permanganate, or after stimula-
tion, fibrous structures as shown in Plate 45 (region of the middle
lamella) or plates 44 or 50 became apparent.

The majority of cellulose micelles of the hinge walls were
aligned parallel to the longitudinal axis of the hair, a fact

supported not only by careful examination of the electron micro-
scope photographs, but also by the fact that sectioning with glass
knives was very much easier if done along the longitudinal axis.

3. NUMERICAL ESTIMATES OF ORGANELLES.

The actual counts of organelles for stimulated and unstimulated hinge cells are given in Tables 1A and 1B, pages 154 and 155. Numbers of mitochondria, plastids and Golgi are given. Table 1C sets out similar counts on certain cells below the hinge region.

Counts for Plate 20 are excluded from statistical analysis, because the section was a "Gold" or thick section. It was included amongst the plates because it showed certain features in the cell wall which occur as a result of bending.

Table 1D shows that the numbers of organelles per 100 square microns are:

Mitochondria	3.13	(Standard Error 0.77)
Plastids	0.29	(" " 0.09)
Golgi	0.16	

TABLE 1A

Organelle counts in stimulated and non-stimulated median; longitudinal sections of the sensory hinge of the Dionaea hair. Cells are numbered from left to right.

A. Non-stimulated hair

Plate no.	Cell	Fixative	Mito-chondria	Plastids	Golgi
12	1-hinge	glutaraldehyde	38	10	2
	2-cond.		30	13	6
	3-cond.		33	13	5
	4-hinge		22	13	12
13	1-hinge	permanganate	63	0	0
	2-cond.		45	8	0
	3-cond.		61	5	0
	4-hinge		74	2	0
14	1-hinge	glutaraldehyde	22	1	2
	2-cond.		17	0	2
	3-hinge		52	0	5

B. Cells in which action potential probably occurred during fixation due to too large temperature difference between specimen and fixative.

15	hinge (RHS)		41	10	0
16	hinge (LHS)		45	13	0

TABLE 1B

C. **Cells fixed in the bent position.** (Fixative was glutaral-
dehyde in all cases.)

Plate no.	Cell	Mito-chondria	Plastids	Golgi
17	1-hinge (compression)	90	1	0
	2-conducting	73	4	0
	3-hinge (extension)	78	4	0
18	1-hinge (compression)	31	3	0
	2-conducting	40	3	1
	3-hinge (damaged)	-	-	-
19	1-hinge (compression)	40	0	6
	2-conducting	48	1	1
	3-conducting	16	1	2
	4-conducting (part)	2	0	0
	5-conducting	43	3	2
	6-conducting	9	1	0
	7-hinge (extension)	52	2	3
20	hinge cell	27	2	0
21	1-hinge (compression)	24	1	0
	2-conducting	47	2	0
	3-conducting	56	1	0
	4-hinge (extension)	38	2	0

TABLE 1C

Counts of organelles in podium below hinge.

	Mito-chondria	Plastids			Golgi
Plate 13					
3 cells below hinge cell 1	9	1 chloroplast S[x]			0
3 cells below cond. cell 2	26	4	"	S	0
3 cells below cond. cell 3	37	5	"	S	0
3 cells below hinge cell 4	11	1	"	S	0
Plate 14					
Cell below cond. cell, 20μ x 20μ	40	3			0
Cell below hinge-compression – 30μ x 20μ	35	1			0
Plate 17					
Cell below hinge-compression	32	2			0
Plate 19					
Cell below hinge cell (Cell only 27μ x 17μ)	50	0			3

All cells below hinge similar.
Highly vacuolate with vesicles.

S This indicated that the chloroplasts contained starch grains.

TABLE 1D

Organelles per unit area (100 square microns).

Statistics have been extracted from tables 1A and 1B. (In these tables, LHS or left hand side is always that of compression, and RHS or right hand side that of cell extension; i.e. the hair was always bent to the left in the hairs represented by plates 17 to 21, which are marked with an asterisk. Other hairs were not mechanically stimulated. Sections were "silver"$_o$, i.e. 600-800 A thick.

Plate no.	Hinge cell, extension or compression	Area in 100 square microns	Organelles per 100 square μ		
			Mitochondria	Plastids	Golgi
12	LHS	11.4	3.33	0.88	0.18
	RHS	14.9	1.48	0.88	0.80
13	LHS	15.9	3.96	0.00	0.00
	RHS	20.4	3.36	0.09	0.00
14	LHS	4.0	5.50	0.25	0.25
	RHS	9.2	5.67	0.00	0.54
15	RHS	11.8	3.47	0.84	0.00
16	LHS	14.7	3.07	0.88	0.00
17*	LHS	33.4	2.69	0.03	0.00
	RHS	36.8	2.12	0.11	0.00
18*	LHS	14.5	2.21	0.21	0.00
19*	LHS	10.2	3.92	0.00	0.58
	RHS	16.0	3.25	0.12	0.19
21*	LHS	18.7	1.28	0.05	0.00
	RHS	25.5	1.49	0.07	0.00
MEAN			3.13	0.29	0.16
S. Error			0.77	0.09	

TABLE 1E

Swelling of mitochondria and appearance of myelin forms.

A. Not stimulated

Hair no.	Total no. of mitochondria	Number swollen	Percentage swollen	Myelin forms
12 LHS*	38	0	0	Nil
RHS*	22	0	0	Nil
13 LHS	63	0	0	Nil
RHS	74	0	0	Nil
14 LHS	22	0	0	Nil
RHS	52	0	0	Nil

B. Probably stimulated by temperature change

15 RHS	41	0	0	Nil .
16 LHS	45	0	0	Nil

C. Mechanically stimulated

17 LHS	90	5	5.6	Forms present
RHS	78	5	6.4	" "
18 LHS	31	1	3.2	" "
RHS	–	–	–	" "
19 LHS	40	12	30.0	" "
RHS	52	14	26.9	" "
21 LHS	24	0	0	" "
RHS	38	0	0	" "

* In all tables LHS (left hand side) of the hinge is the side of compression, and RHS (right hand side) is the side of extension. That is, in all cases the hair was bent to the left.

TABLE 1F

Analysis of variance for the <u>number</u> of mitochondria, according to <u>position</u> (top, middle or bottom of the hinge cell).

The method of dividing the hinge cell was the same for each cell – the maximum distance parallel to the longitudinal axis was divided into three equal portions by two horizontal lines.

For each section, LHS or left hand side was the side of compression. RHS or right hand side was the side of extension.

There were two types of hair measured, under the general term <u>touched</u>. These types were unstimulated, or stimulated by bending the hair, and fixing it in this position with glutaraldehyde.

The sums of Squares are from data transformed from a Poisson to a Normal distribution, according to the formula $\sqrt{x + \frac{1}{2}}$, where x is the actual number of mitochondria. Certain details are given in the following page, Table 1G.

Analysis of variance

Source	S. Squares	Degrees freedom	Mean Square
Between plants			
Stimulation	2.834	1	2.834
Plants error	18.171	4	4.543
Within plants			
Side (LHS or RHS)	0.562	1	0.562
Side x Stimulation	0.967	1	0.967
Region	30.504	2	15.252[a]
Region x Stimulation	6.597	2	3.298
Side x Region	1.086	2	0.543
Side x Region x Stimulation	0.833	2	0.416
Pooled error[b]	55.176	20	
Total	116.730	35	

[a] This is the only significant result. That is, the mitochondria were concentrated in a particular region.

[b] The pooled error is derived by pooling the errors from Region x Plants and Side x Regions x Plants, as well as Side X Plants.

TABLE 1G

Figures extracted from the Analysis of Variance on Table 1F, the previous page. The figures for transformation from a Poisson to a Normal distribution are given below. That is, the actual number of mitochondria (x), have been transformed according to the formula $\sqrt{x + \frac{1}{2}}$.

"Stimulated" refers to hairs fixed in the bent position. The plate numbers refer to sections of hairs represented by drawings shown in plates 12,13,14,17,18 and 19 on pages 67,69,71, 77,79 and 81.

Hinge cells

No. of mitochondria (transformed values)

	Top	Middle	Bottom	Percent in middle
Not Stimulated	16.9	31.3	12.5	51.6
Stimulated	21.1	28.0	21.7	39.5
TOTALS	38.0	59.3	34.2	45.1

The transformed data from which Table 1F and the table above were derived, are given below. Only complete, median, longitudinal sections of the same thickness have been included in the Analysis of Variance. LHS is the side of compression, and RHS that of extension of the hinge.

Not Stimulated		Top	Middle	Bottom
Plate No. 12	LHS	3.4	4.5	2.1
	RHS	3.9	2.7	0.0
13	LHS	3.5	7.1	2.1
	RHS	0.0	8.7	2.1
14	LHS	1.9	3.8	2.3
	RHS	4.2	4.5	3.9
Stimulated				
17	LHS	3.2	7.5	5.0
	RHS	4.3	5.6	5.4
18	LHS	4.2	2.3	2.3
	RHS	2.9	4.7	4.7
19	LHS	3.5	3.2	1.6
	RHS	3.0	4.7	2.7

In regard to Golgi, six out of the nine hairs of which a sample was studied, showed no Golgi at all.

Table 1E, page 158, showed that in sections from unstimulated hairs there were no swollen mitochondria, or myelin forms, but when the hairs were bent, myelin forms always occurred, and readily visible swollen mitochondria were seen in samples of three hairs out of four.

Table 1F sets out an analysis of variance, to ascertain whether indeed the mitochondria were concentrated at the middle region, and whether there was any movement of mitochondria with bending. For this analysis, the hinge cells were divided in the same way, into three portions, top, middle and bottom, and the mitochondria counted.

The significant item of Table 1F was that in both stimulated and non-stimulated hairs, there was a concentration of mitochondria in the middle region of the hinge. There appeared to be some movement of mitochondria away from the mid-region with bending, but with the specimens analysed the degree of movement did not reach significance. These two points are emphasized in Table 1G, and further studies of mitochondrial movement with stimulation are warranted, using data from more samples per hair, and more hairs.

5. Recovery of "damaged" hinge cells.

On six occasions, an intact plant was taken, and the sensitive hair was bent down almost parallel to the lamina, at 0°, 180°,

90° and 270°, so as to cause the type of injury shown in Plate 18, pages 77-78. This ill-treatment was repeated for 3 consecutive days. After each treatment, the trap closed, but reopened within 24 hours, and the "damaged" hair was as sensitive as ever.

6. Main changes in fine structure with stimulation.

The main changes which occur in fine structure, are those shown by DANIEL, DINGLE, GLAUERT and LUCY (1966), as resulting after treatment of rat fibroblasts with excess vitamin A alcohol (retinol). These are alterations in the membranes of the cells, distending the plasma and other membranes, causing gross swelling of mitochondria.

Golgi and cisternae distend and become indistinguishable, and myelin-like structures composed of concentric layers of membranes are seen in rat cells treated with excess Vitamin A, and as a result of bending the sensory hair of Dionaea.

C H A P T E R   7

D I S C U S S I O N   O F   R E S U L T S

F I N E   S T R U C T U R E

DISCUSSION OF RESULTS

1. A. Comparison with animal sensory organs.

The most notable feature of the fine structure of the "hinge" cells, and those immediately below in the podium, are the concentration of mitochondria and vesicles, in common with animal sensory receptors, described by PEASE and QUILLIAM (1957) for the Pacinian corpuscle, by SMITH and DEMPSEY (1957) for the organ of Corti, by FARBMAN (1965) for the taste bud, by ENGSTROM and SJOSTRAND (1956) for the cochlea hair cells, by KARLSSON (1966) for certain neurones of the rat, SJOSTRAND (1953) for the retinal rod, and by numerous other research workers for a variety of sensory organs, the nerve-muscle synapse, certain of which have been mentioned in the Review of the Literature, pages 32 to 34.

The other notable features are the extensive ER and vacuoles, and the type of plastid.

B. Comparison with the fine structure of the electric organ of the electric eel, and of electric fish.

The sensory hinge of Dionaea, in accumulating an electric potential (JACOBSON, 1965) can be likened to an electric organ of animals.

LUFT (1958) described in the electroplaques of Torpedo and Electrophorus "a highly developed system of caveolae, or cave-like blind tubules, and vesicles." According to him, the caveolae are blind, elongate, sac-like indentations of the plasma membrane of

the cell. He also described as associated with the system, completely intracellular vesicles of approximately the same diameter as the caveolae. MATHEWSON et al (1961), and WACHTEL et al (1961) compared the electroplaque of fishes with the neuro-muscular synapse.

SHERIDAN (1965) noted in the dorsal third of the electroplan of Torpedo marusata, a tubular membranous network whose lumen is continuous with the extracellular space. Mitochondria are present, and nerve endings, separated from the ventral surface of the electroplaque by a thin membrane, contain synaptic vesicles (diameter 300 to 1200 A), mitochondria, and electron-opaque granules (diameter 300 A).

2. Comparison of the fine structure of the hinge cells of Dionaea with that of certain animal excretory cells.

Certain features common to many secretory tissues of animals have been reviewed in the discussion by LENNEP and LANZING (1967) in their discussion of the dendritic organ of marine catfish. Certain features in common were noted in organs concerned with the transport of water and inorganic ions, such as kidney tubules, striated ducts in salivary glands, choroid plexus, and salt glands in marine birds. Also in certain species of fish where sodium chloride is either excreted from the gill filaments of teleosts, or in other species from rectal glands, all show features common to those organs already listed in this paragraph. These features are invaginations

of the basal plasma membrane (with the exception of teleost gills, where a well-developed ER is in the form of a tubular network which is connected with the basal plasma membrane), and the alignment of numerous mitochondria between them. The plasma membranes are very extensively folded, as is the case in Dionaea hinge cells' ER.

VAN LENNEP and LANZING (1967), in discussing the fine structure of the dendritic organ of some catfish and the probable salt secreting function of this organ, discussed their use of the term smooth endoplasmic reticulum, which cannot be compared with that of adrenal cortical cells or corpus luteum, but must be regarded as extensions of the plasma membrane and their lumina as extracellular space. There was a notable absence of Golgi apparatus, and communication between the "smooth ER" and granular ER was never observed.

3. Comparisons with certain plant organs.

In the literature review, pages 35 to 37, it was shown that in Nitella, in active phloem, in raphide-forming cells and in the digestive gland of Dionaea, there is a well developed ER; moreover, raphide secreting cells have a type of plastid similar to that observed in the hinge of Dionaea.

A further comparison can be made from the work of CHAMBERS and MERCER (1964) with Chara, where there was abundant ER, which was linked occasionally with the mitochondrial and nuclear membranes, as well as to the chloroplast. ATKINSON et al (1965) noted that in the salt secreting glands of two genera of mangrove, as well as the

salt gland of <u>Avicennia marina</u>, there were a thick cuticle, many vacuoles and many mitochondria in the sub-basal cells near plasmodesmata. They noted that the discovery of the mechanism of salt pumps in these glands awaits further investigation.

4. <u>Possible significance of the plasmodesmata in the hinge relative to sensitivity</u>.

An examination of serial sections of all hairs studied, both stimulated and not stimulated, reveals that there is an abundance of plasmodesmata frequently about 100 mμ in diameter, radiating from the hinge cells to the inner "conducting" cells, as well as to the cells above and below the hinge region; and which are sometimes found in the thick cell wall in the zone of hinge cell indentation (plates 23, 33 and 34).

Reference to Plate 2, page 15 shows that the ratio of the length of the lever to the length of the hinge is approximately 12/1. ~~Because the hair is relatively narrow at its base, there is little mechanical advantage when lateral movement of the hair at its tip occurs.~~

The length of the sensory hair, and the positioning of these hairs (usually six in number), is such as to insure that the hinge region is most likely to be stimulated by an insect such as a fly.

There is evidence that both the animal Pacinian corpuscle (DUNCAN, 1967) and the sensory hair of <u>Dionaea</u> (LLOYD, 1942 and JACOBSON, 1965) function enzymatically. DUNCAN (1967) stated that

for the Pacinian corpuscle that stimulation of a very small area, as by a stylus, produced a localized current in the receptor, which decreased exponentially with the distance from the point of stimulation, and that increasing the stimulus strength or area of stimulation produced a progressively greater degree of deformation and the excitation, measured with a microelectrode, spread over a correspondingly greater area.

Copious ramification in all planes of plasmodesmata from the hinge cells through the cell walls where bending is known to occur most readily, could be expected to ensure the maximum area of stimulated outer membrane, and hence maximum excitation for a given stimulus.

5. Effects of stimulation on fixation.

SIBAOKA (1966), JACOBSON (1965) and others have compared action potentials in plants with those of a nerve. MACROBBIE (1962 and 1966) and RAVEN (1967 b) have discussed a sodium-potassium pump, as occurs in animal tissue, whilst RAVEN (1967 a and 1968) has discussed a light-stimulated chloride ion pump involving the net salt uptake mechanism. The above work will be discussed in detail in later sections.

By comparison with animal receptor organs (DUNCAN, 1967), stimulation could be expected to lead to an alteration in the ionic conditions, both in the cell walls, and in the hinge cells themselves. FINEAN (1968) indicated the dearth of knowledge in regard

to the true nature of fixation and post-fixation with osmic acid, and staining with heavy metals. An alteration in ionic conditions, expected to occur after an action potential, could explain some of the changes, such as the greater degree of staining of the cell walls and the lack of clearness in the appearance of membranes, in all cases where stimulation of the sensory hair of Dionaea has occurred.

Alterations in pH alone are unlikely to cause alteration in clearness of the membranes, since SCHULTZ and KARLSSON (1965) have studied the effects of pH during fixation with 2.5 per cent glutaraldehyde, followed by post-fixation with osmic acid, at a range of pH between 2 to 11. These workers found that the appearance of membranes were fairly clear and sharp at these extremes.

6. Stimulatory change in relation to Vitamin A studies.

Reference to Table 1E, page 158 shows that in all five hairs where mechanical bending had not occurred, there were no swollen mitochondria or myelin forms. In the four hairs which were mechanically stimulated, myelin forms occurred in each case, and swollen mitochondria in the hinges of three hairs out of four.

MITCHELL (1967) found localized swelling in mitochondria of ascite tumor cells sometimes occurred when there was a significant drop in pH, and since the closing of the traps of Dionaea is based on turgor changes (STUHLMAN, 1950), stimulation might be expected to lead to a sudden drop in turgor, and to swollen mitochondria.

The occurrence of swollen mitochondria in most hairs examined,

with the presence of myelin forms (Table 1E) in bent hairs, compared
with normal mitochondria and an absence of myelin forms. These
results can be related to the studies of LUCY and DINGLE (1962 and
1964), and DANIEL, DINGLE et al (1966), already reviewed on pages
37 and 38 of this study.

To emphasize certain of these results, LUCY and DINGLE (1962)
suggested that Vitamin A in small quantities (up to 6 μg/ml) might
alter membrane permeability by activating an enzyme system which
can attack the membrane, or by altering membrane structure, or inter-
fering with membrane synthesis. ROELS et al (1965) suggested
similar mechanisms for the action of Vitamin A on the lysosomal
membrane.

DANIEL, DINGLE et al (1966) found that tissue treated with
excess Vitamin A alcohol showed in general, a similar appearance to
that of the hinge of Dionaea after stimulation – distension of mem-
branes and swelling of organelles (compare especially plates 23 to
40 representing unstimulated hinge cells with plates 41 to 52
representing stimulated hinge tissue, and notice ER, mitochondria
and plastids), and the occurrence of myelin forms.

GLAUERT et al (1963) have suggested that the action of excess
of Vitamin A on erythrocyte membranes is a re-orientation of the
structure of the membrane. This concept is supported by the
explanation of LUCY and DINGLE (1964), based on the LUCY (1964)
membrane model, that a membrane containing a large quantity of

Vitamin A alcohol may have a high proportion of its structure in the more permeable micellar form, and this concept will be reviewed in much more detail in a later chapter.

CHAPTER 8

RESUME OF RESULTS
FROM FINE STRUCTURE

are discussed. The association between the hair of Diadema be just stated, can recharge in like minutes.

The hair of Diadema was also found to extend considerable charge and retain its sensitivity to Bohin being, possibly indicating the ability of the hinge ends to recover from injury quickly.

Despite the suggestion of Blank et al (1968 b) that the

RESUME OF RESULTS FROM FINE STRUCTURE

As shown in tables 1A, B, C and D, pages 154 - 157, there is a high concentration of mitochondria (3.13 mitochondria per 100 square microns) in the hinge cells. As shown in plates 12 to 21 inclusive, and illustrated in Table 1F, page 159, there is a concentration of mitochondria in the horizontal plane of the hinge furrow. This is seen, for example, in Plate 22. Another feature of the hinge cells is the large number of vesicles, and these two factors, large numbers of mitochondria and vesicles, are common to the animal neuron, the nerve-muscle synapse, and other animal receptor organs reviewed on pages 32 to 34 of this study.

The hinge cells also contain abundant ER, and this is found in animal and plant cells where very active internal or external secretion occurs, reviewed on pages 35 to 37. Further examples are given in the discussion section, where salt secreting and electro-plaque tissues of animals, and salt secreting cells of plants are discussed. The association between ER and very active transport is understandable in view of the fact that the hinge of Dionaea, as just stated, can recharge in five minutes.

The hair of Dionaea has been found to stand considerable abuse and retain its sensitivity 24 hours later, possibly indicating the ability of the hinge cells to recover from injury quickly.

Despite the suggestion of SCALA et al (1968 b) that the

plastids might be involved as capacitators of the electric charge, Table 1D, page 157, indicates a low frequency of these organelles, and the similarity to the plastids of <u>Vanilla</u> and <u>Monstera</u>, where a lipid secreting function has been suggested by MOLLENHAUER and LARSON (1966), is more in keeping with the fact that the hinge cells of <u>Dionaea</u> contain many lipid droplets.

The occurrence of numerous small plasmodesmata, ramifying all the walls of the hinge in all planes, is functionally very significant, in increasing the area of membrane distorted, the significance of which has been reviewed by DUNCAN (1967) in regard to the Pacinian corpuscle.

The appearance of the fine structure of the hinge of <u>Dionaea</u> in the bent position resembles certain alterations similar to those found by DANIEL, DINGLE et al (1966) for tissue treated with Vitamin A alcohol:- distension of membranes and swelling of organelles, as well as the appearance of myelin forms. The alteration of the structure of the phospholipids in the membrane from the globular micelle, to the bi molecular leaflet and more permeable form, as indicated in the LUCY (1964) model, has been suggested by LUCY and DINGLE (1964) and GLAUERT et al (1963), for this type of membrane alteration.

DANIEL, DINGLE et al (1966), and ROELS et al (1965) have suggested similar mechanisms for the action of Vitamin A alcohol on the erythrocyte and lysosome membrane permeability respectively,

as follows:-

1) Vitamin A may increase the activity of an enzyme capable of attacking the membrane.

2) The vitamin may directly alter the structure of the membrane.

3) The vitamin may interfere with reactions essential to the maintenance of the membrane.

CHAPTER 9

PHYSIOLOGICAL EXPERIMENTS

PRELIMINARY INTRODUCTION

THE MAIN POINTS TO BE DISCUSSED ARE:

THE SIMILARITY OF FINE STRUCTURE OF THE SENSORY HINGE OF DIONAEA
TO THAT OF THE NERVE-MUSCLE SYNAPSE, IN WHICH ACETYL-CHOLINE IS A
CHEMICAL TRANSMITTER.

THE SEARCH FOR ACETYL CHOLINE AND CHOLINESTERASE IN THE TRAPS OF
DIONAEA, AND THE DISCOVERY OF A MUSCLE CONTRACTING SUBSTANCE (MCS),
WHICH CONTRACTS FROG RECTUS MUSCLE, AS DOES ACETYLCHOLINE.

THE ASSAY OF THIS MCS IN THE TRAPS OF DIONAEA, UNDER DIFFERENT
CONDITIONS OF STIMULATION, AND TIMES AFTER MECHANICAL STIMULATION.

IDENTIFICATION OF THIS MCS AS LYSOPHOSPHATIDIC ACID BY DIFFERENT
TECHNIQUES, AND ITS USE AS A MEASURE OF PHOSPHOLIPASE ACTIVITY.

THE EFFECTS OF CHLOR-CHOLINE-CHLORIDE, CONSIDERED TO STIMULATE
LECITHIN PRODUCTION, ON THE MECHANICAL STIMULATION OF THE DIONAEA
SENSORY HINGE.

INTRODUCTION

Now that the details of fine structure of the sensory hinge have been covered in the preceding chapter, certain features in common with the nerve-muscle synapse, that is numerous vesicles and mitochondria, referred to in the General Introduction on pages 13 and 14, have been made evident.

The discovery by EMMELIN and FELDBERG (1947), referred to on page 33, of relatively large quantities of acetylcholine, suggested looking for acetylcholine in the sensory hair and traps of Dionaea, using the methods described by PERRY et al (1968), where the quantity of acetylcholine is measured by the degree of contraction of the rectus muscle from the abdomen of a frog.

Since a muscle contracting substance was found, and identified as being fairly definitely lysophosphatidic acid, when the traps of Dionaea had been ground up in frog Ringer's solution and incubated for one hour, there was good evidence that the mechanism of the action potential from the sensory hair was to activate a phospholipase, probably a phospholipase D.

Chlor-choline-chloride (CCC) was used in solution on detached whole leaves with their traps undisturbed. The bases only of these whole leaves were immersed in varying concentrations. At first ccc was used as an analogue of acetyl-choline, but later evidence for its effect on lecithin production, and hence on membrane synthesis, was found.

In order that the final discussion can be wielded deftly, the Review of the Literature which now follows involves all aspects of the molecular properties of excitable cells; bioelectrochemistry; action potentials in plant organs, membrane structure; membrane permeability and phospholipase action, especially in regard to the role of calcium; the effects of CCC on lecithin production; and evidence from bacterial research that a permease may be a phospholipase.

The molecular properties of excitable cells.

A. Animal cells.

B. Plant cells.

A. Animal cells.

Theories for the transducer process of animal cells have been reviewed by DUNCAN (1967), and are summarized below.

DUNCAN based his appreciation of the mechanoreceptor on the review by GRAY (1959).

a) Mechanoreceptors.

i) The action of chemical transmitters.

Both mechanoreceptors and end-plate potentials produce an action potential, and acetyl choline has been suggested as an intermediary in the excitation of sense organs. The evidence suggests that it is most improbable that acetyl choline acts in this way, ~~in sense organs~~ (DUNCAN, 1964).

ii) Distortion* of membrane area.

The stretching* of the bounding membrane of the receptor could modify the permeability of the membrane directly, possibly by a simple mechanical deformation of the "pores," as observed by HUBBARD (1958) with the Pacinian corpuscle.

Coupled with this hypothesis, is the suggestion that mechanical displacement produces a change of pressure which causes certain changes in molecular organization, either of the membrane itself or of hydrated ions on either side of it (GRAY, 1959).

* 'Distortion' &'stretching' are used in the sense of "increase in area."

The high degree of mechanosensitivity of the Pacinian corpuscle is confined to the length of about 700 µ of the non-myelinated nerve ending. Other regions of the sense organ are relatively insensitive (LOEWENSTEIN, 1961 b).

DUNCAN (1967) stated that experiments with mammalian Pacinian corpuscles and Amoeba can be interpreted on the basis that an enzyme localized in the receptor membrane is involved. It is the area of membrane involved which determines the size of the receptor current. This interpretation is based especially on the area stimulation results of LOEWENSTEIN (1959, 1961), and temperature studies by ISHIKO and LOEWENSTEIN (1960, 1961).

b) Animal sense organs generally.

i) The action of chemical transmitters.

DUNCAN (1967) concluded that certain cholinesterase inhibitors in high concentration are able to inactivate certain sense organs. He considered that acetylcholine is not used in the transducer mechanism of sensory receptors, rather, DUNCAN suggested that a non-specific cholinesterase activity is associated with the ATP ase enzyme complex, which governs cation-permeability.

DUNCAN presented evidence to show that various ATP ases are linked with cholinesterase in this way, and that their ATP-hydrolysing activity can be modified by cholinesterase inhibitors.

ii) In summarising the concensus of hypotheses for axon and receptor cation-permeability changes, DUNCAN (1967) stated that in

the axon the action potential released Ca^{2+} affecting permeability, and this permeability is augmented by the removal of the inhibitory calcium ions from the ATP ase controlling a contractile protein. Receptors of touch, light, taste and smell all have their effects on the ATP-ase contractile protein mechanism of permeability change.

DUNCAN (1967) stated that all animal sense organs are temperature-sensitive, reflecting their dependence on enzymatic transducer mechanisms. Thermoreceptors have evolved by an increase in temperature sensitivity. ASHIDA (1937) found with Aldrovandra that sudden temperature changes (he used changes of $10^{\circ}C.$) in either direction would cause closure. As already described, the work of BROWN and SHARP (1910) with Dionaea traps concerning the number of stimuli necessary and temperature, indicated an enzymatically controlled process between receptor and effector.

To summarise clearly and briefly, the concensus of evidence reviewed by DUNCAN (1967) is that all animal sensory mechanism is due to an action potential from the organ itself. This action potential is operated enzymatically, involves an ATP ase which causes a contraction in the protein outer layers of the membrane, and the alteration in the molecular configuration and/or charge distribution at the membrane pores. This produces a change in the ionic permeability of the excitable membrane. Mechanoreceptors represent the simplest example of such a system, and mechanical deformation of the membrane produces a direct modification of the

relationship between the membrane enzyme and its substrate.

B. Plant cells.

  a) Tactile response of tendrils.

  JAFFE and GALSTON (1968) have proposed the following hypo-
thesis to explain the tactile response of tendrils:- The touch
stimulus on the ventral surface elicits an action potential which
causes the activation of an ATP ase which affects a contractile
protein in the membrane of the tendril, resulting in the contrac-
tion of the ventral surface and an expansion of the dorsal surface.
The contractile-ATP ase system may be associated with chemi-osmotic
coupling, in a pumping mechanism which pushes solutes out of the
cells on the ventral side. If this occurs, water would flow out of
these cells, causing the observed ventral cell shrinkage.

  b) Photo-receptors and geo-receptors in plants.

  Whilst hypotheses for the action of light and gravity in
bringing about movements in plants have an auxin basis, the recog-
nition by JUNIPER et al (1966) that the perception of gravity
existed in the root cap of certain plants, suggests that the stimu-
lus to the zone of cell elongation in the root is possibly trans-
mitted by means of an action potential.

  c) Phytochrome and membrane permeability.

  The results of FONDEVILLE et al (1966), of FONDEVILLE et
al (1967), and JAFFE and GALSTON (1967) with Mimosa pudica and
Albizzia julibrissin, have raised the question whether phytochrome

is part of the membrane, and the action of far-red light is to decrease permeability, whilst red light may increase permeability.

Possible relationship between the sensitive hair and trap of Dionaea and the nerve-muscle synapse of animals.

DALE (1914) first suggested that a choline-ester, probably acetylcholine, was the chemical transmitter in the animal parasympathetic system. This was later proven by LOEWI (1921), who demonstrated that the vagus nerve inhibits the heart by means of acetylcholine. The chemical transmitter hypothesis was extended to the sympathetic ganglia and neuromuscular junction, and has become a well-established theory now.

EMMELIN and FELDBERG (1947) showed that in the hair of the stinging nettle, Urtica urens there were relatively large quantities of acetylcholine (between 0.27 and 0.18 μ g per mg leaf tissue), serotin (5-hydroxytryptamine), and histamine, but that it did not contain choline esterase. Each of the three substances found is known to stimulate nerve tissue in very small quantities. The structure of the sensitive hair of Dionaea has a fine structure similar to that of the nettle, as already noted.

The manner in which acetyl choline is considered to be produced and broken down in the nerve-muscle synapse is described by NACHMANSOHN D. (1959):-

acetate + choline $\xrightarrow{\text{acetylase}}$ acetyl choline $\xrightarrow[\substack{\text{esterase} \\ \text{inhibited by} \\ \text{eserine}}]{\text{acetylcholine}}$ acetate + choline

$\downarrow$ contracts rectus muscle of frog
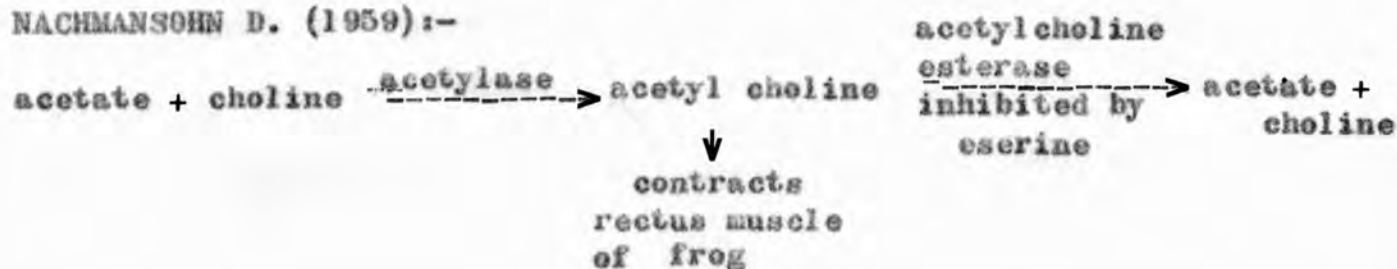
BIOELECTROCHEMISTRY

Introduction

Stimulation of the sensitive hair of <u>Dionaea</u> by a slight touch evokes an action potential, as recorded by JACOBSON (1965), DARWIN (1875), and others. STUHLMAN (1950) linked closure of the trap specifically to a sudden reduction in the hydrostatic pressure of the cells of the inner epidermis. Such a loss of turgor in the epidermis can be explained best by a sudden loss in permeability of the plasmalemma, which itself would be accompanied by an alteration in electric potential. Both the restoration of the potential to the hinge cells of the trigger hair, and of turgor to the re-opening trap, requires an understanding of pumping mechanisms, and of bioelectrochemistry generally, and so the following relevant inform-ation is reviewed.

Any relationship of plants to the transfer of electrons qualifies for consideration under bioelectricity. According to this concept, all oxidations and reductions associated with plants are included, since these reactions involve electron transfer. Potential differences exist where there are differences in the concentration of ions, and such potential differences appear across all membranes. The energy involved in active transport of ions to produce such differences can be expressed in electrical terms, and can be measured electrically, facts known by very early workers such as Luigi GALVANI (1791), who published his work with animal electricity; by DARWIN (1875), who measured potentials involved with the action of the sensitive hair of Dionaea; and EHRLICH (1885), who published monographs which laid the basis for the measurement of redox potentials with dyes.

Active ion transport.

The vacuoles of plants accumulate ions vastly in excess of their external concentrations. STEWARD and SUTCLIFFE (1959) have listed typical "accumulation ratios" for both algae and higher plants. The chloride and the potassium accumulation ratios may each exceed 1000 to 1.

A second major consideration is that cells of higher plants possess membranes which have a very low permeability to free ions (ARISZ, 1964). This fact is inherent in the Mitchell hypothesis, even in reference to H^+ and OH^- ions (MITCHELL, 1961), where

large differences in pH can exist between the inside of the mito-
chondria, chloroplasts, and cytoplasm of an individual cell. The
Mitchell hypothesis will be discussed in more detail in the
Discussion, following results.

Injury or even mild treatment such as withdrawal of calcium,
causes damage to membranes, and impairment of their ability to
retain ions (FOOTE and HANSON, 1964).

The third major point, is the remarkable selectivity possessed
by ion-transporting membranes. Thus the rate of potassium accu-
mulation by barley roots may be completely unaffected by the presence
of much higher concentrations of sodium in the external solution.
EPSTEIN (1961 & 1962) has discussed numerous such instances.

Correlations between ion transport and metabolism.

Ion transport can be interfered with by darkness (MACROBBIE,
1962), by low oxygen tensions (HOAGLAND, 1944) and by metabolic
poisons (ORDIN and JACOBSON, 1955). The exposure of plant tissue
to salt solutions has often been observed to induce an increase in
the rate of respiration, such increase being sensitive to cyanide,
light (reversibly) and to carbon monoxide.

LUNDEGÅRDH (1960) proposed that since four electrons are
involved for a molecule of O_2, and the ratio, moles monovalent ions
transported / moles O_2 consumed in the induced respiration,
approaches but never exceeds 4, that anions travel along the chain
of alternately oxidised and reduced members of the cytochrome

chain, being initially caught by cytochrome oxidase.

Difficulties have been found with this hypothesis, because cytochrome of the respiratory chain is in the mitochondria, and 2-4 dinitrophenol (DNP) increases the rate of respiration of carrot discs but inhibits salt absorption (ROBERTSON et al, 1951), thus indicating that oxidative phosphorylation coupled electron flow, and not electron flow itself, is involved in ion transport. EPSTEIN et al (1963) has found evidence for more than one mechanism for absorption of potassium by barley, but the most convincing evidence against the LUNDEGÅRDH hypothesis is that of BUDD and LATIES (1964), who found that with corn roots under anaerobic conditions, the electron flow could be maintained over a shortened portion of the cytochrome chain by the use of ferricyanide as an electron acceptor, and found that the metabolic transport of chloride under such conditions was similar to aerobic transport.

The role of phosphorylation.

  1. Animal tissues.

For animal tissues, the knowledge of the mechanism for the exclusion of Na^+ and accumulation of K^+ in cells is rudimentary, but there is strong evidence that the enzyme system known as 'Na^+ and K^+ dependent adenosine triphosphatase' is closely associated with this mechanism. The salient characteristics of this mechanism in animals are:-

  1) The enzyme system is membrane bound and everywhere in

animal cells.

2) It requires Na^+ and K^+ ions for activation.

3) In the erythrocyte, enzyme activation occurs only when Na^+ is on the 'inside' and K^+ is on the 'outside' of the cell.

4) Na^+ and K^+ activated ATP ase is specifically inhibited by the cardiac glycoside ouabain, known to be a potent inhibitor of cation transport both in vivo and in vitro.

5) Kinetics of the reaction in vitro are such that activation by substrate and ions, and inhibition by ouabain are at concentrations compatible with physiological conditions.

Studies by CHARNOCK and POST (1963) and others have suggested the following reaction mechanism for the hydrolysis of ATP by an ATP ase, in the presence of Mg^{++}, Na^+ and K^+ :-

$$\text{ATP Enzyme} \underset{(K^+ \text{ stimulated})}{\overset{(Na^+ \text{ stimulated})}{\rightleftharpoons}} E \sim P + ADP \quad (1)$$

$$E \sim P + H_2O \qquad\qquad E + Pi.$$
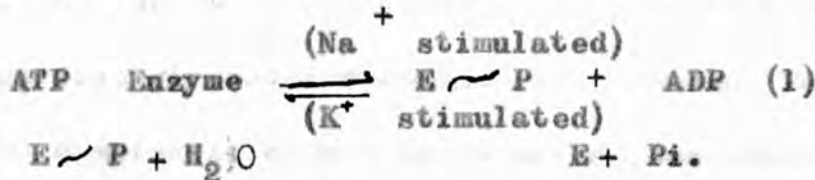
OUABAIN (CARDIAC GLYCOSIDE)
INHIBITS

where E = enzyme, and Pi = phosphate.

The chemical nature of the phosphorylated complex $E \sim P$ is almost certainly a phosphoprotein.

The significance of this mechanism, is that it is an integral part of the system in animals and plants whereby Na^+ ions are actively pumped out, and K^+ ions are pumped into cells. In animal nerve tissue, it is the sudden breakdown of permeability of

the plasma membrane which leads to a sudden inflow of Na^+ and

outflow of K^+ ions, and an action potential

$$\text{ATP + enzyme} \overset{Na^+}{\rightleftharpoons} E \sim P + ADP \qquad (1)$$

$$E \sim P + H_2O \xrightarrow{\underset{OUABAIN}{\overset{K^+}{\uparrow}}} E + Pi \qquad (2)$$
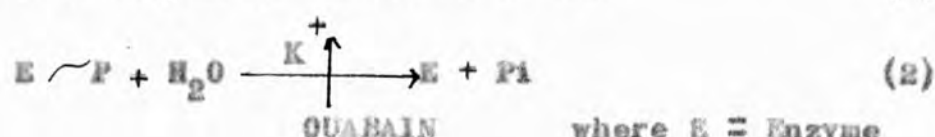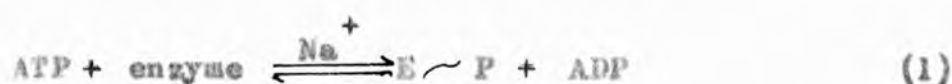
where E $=$ Enzyme

Pi$=$ Phosphate

The initial reaction step consists of the rapid reversible

formation of a phosphorylated complex, stimulated by Na^+ ions, but

no other ion appears able to replace Na^+. The formation of this

complex is followed by an almost irreversible decomposition to

phosphoric acid and free enzyme. This latter reaction is stimulated

by K^+ but not Na^+ ions; K^+ can be replaced with either NH_4^+,

Li^+, Rb^+ or Cs^+. It is this decomposition which is inhibited by

the cardiac glycoside ouabain. The chemical nature of the phosphor-

ylated complex is almost certainly a phosphoprotein.

A hypothetical model for the directional nature of the trans-

port has been suggested by OPIT and CHARNOCK (1965).

These results from animal tissues have led to a search for

similar relationships in plant cells. In the alga Nitella

translucens, MACROBBIE (1962) has found that influx of potassium and

efflux of sodium are sensitive to ouabain, but absorption of chloride

is not. Such mechanisms of ouabain-sensitive ATP ases have been

shown for yeast and certain algae especially by RAVEN (1967a and

1967b) on the ionic relationships of Hydrodictyon africannum and

by MACROBBIE (1965, 1966) in Nitella. This $K^+ - Na^+$ pump can be sup-
ported by cyclic phophosphorylation (MACROBBIE, 1965, 1966; RAVEN,
1967b). At least the Na^+ efflux component appears to be found in
all the algae (RAVEN, 1967a) and higher plants (HIGINTHBOTHAM,
ETHERTON and FOSTER, 1967; PITMAN and SADLER, 1967) that have been
studied. OERTLI (1967), working quite independently and with the
uptake of rubidium by excised barley roots, explained uptake by two
mechanisms, one actively pumping salts in, the other a passive or
"leak" process.

The other mechanism, apparently responsible for maintaining
the total ion centration in the cell higher than outside, is an
ouabain insensitive chloride pump (RAVEN, 1967a). This chloride
pump which seems to involve the net salt uptake mechanism, is light-
stimulated (RAVEN, 1967a). RAVEN (1968) found that the Cl^--dependent
portion of the K^+ and Na^+ influxes have similar responses to changes
in photosynthetic metabolism; both are supported more efficiently
by shorter wavelengths (670 nm) compared with longer wavelengths
(725 nm), correlated with a greater efficiency of Photosystem 2.
D.C.M.U., that is 3'- (3,4- dichlorophenyl), 1'1' dimethylurea,
at 10^{-7}M is a selective inhibitor of Photosystem 2, and inhibits
the ouabain-insensitive K influx, both in the presence and absence
of Cl^-; this ouabain-insensitive K^+ and Cl^- influx component is not
inhibited by the uncoupler C.C.C.P., that is, by carbonyl cyanide m-
chlorophenyl hydrazone at 5×10^{-6}M (RAVEN, 1968). Such a

light-stimulated Cl⁻influx pump is also present in <u>Nitella</u>
(MACROBBIE, 1962, 1965, 1966).

<u>To summarise bioelectrochemistry</u>:-

There are two major but distinct pumping mechanisms in plants.
One is a sodium-potassium pump at the plasma membrane, and is ouabain
sensitive. The other is an ouabain insensitive chloride pump,
light-stimulated, and seems to involve the net salt uptake mech-
anism. A sudden alteration in membrane permeability can lead to an
action potential, caused by the sudden release of ions which have
been pumped into the cell.

Action potentials and potential changes in plant organs.

The situation has been reviewed by SIBAOKO (1966), who discussed three kinds of plants, traps of Dionaea, pulvini of Mimosa, and the cells of the Characeae. In all three kinds of plants, the electrical response of the excitable cells is an all-or-none action potential and not a graded local potential, with a "memory" of subthreshold stimuli which can give an accumulated response, and is not understood.

In all three types of plants, there is no evidence of a chemical transmitter in association with the action potential, except in Mimosa, where as well as a) the "moderately rapid" conduction of stimulus caused by an action potential, there are b) rapid conduction, occasionally observed in the young leaf following a cutting stimulus, in which no electrical changes are associated, and its mechanism has not been explored, and c) a very slow stimulus, having a negative electric potential ~~in shape~~, and long in duration, correlating with the movement of the stimulant in the vessels. This passes through a dead zone, and the rate of propagation depends upon the velocity of the water current in ~~vessels~~ vascular bundles. (SIBAOKO, 1953).

In Mimosa, the rate of spread of the action potential depends on temperature, and between 20 to 30°C. it has a Q 10 of 2.3 to 3.9, being a larger Q 10 in younger tissue. Transmission of the action potential in Mimosa is along the parenchyma cells of the protoxylem and phloem, which parenchyma is itself excitable.

In all three plants, the plasma membrane is polarized, the interior being about 100 millivolts negative to the exterior, and during activity this potential charge becomes reduced in the depolarizing direction.

UMRATH (1939) found that tactile stimulation in cucumber tendrils causes an action potential. JAFFE and GALSTON (1968) consider it probable that an action potential triggers contact coiling in tendrils.

Changes in potentials have been recorded also in higher plants by SCOTT (1962), associated with the downward movement of I.A.A. applied to decapitated oat coleoptiles, and in roots in which the bathing electrolytes are charged.

WILKINS and WOODCOCK (1965) showed that whether a plant shoot was placed in the horizontal position, or whether only a decapitated shoot with 0.02 per cent indolyl - 3 - acetic acid solution in anhydrous lanolin applied to the top of the segment was ~~similarly~~ placed vertically, a change in potential difference across an individual segment occurred in the same pattern, regardless of whether the auxin gradient occurred as the result of geotropic stimulation, or of applied auxin. About 15 minutes after the auxin stimulus, the lower side became electronegative with respect to the upper side, and the potential difference continued to increase for about 20 minutes. A difference of at least 10 mV was maintained for at least a further 20 minutes, and then the gradient decreased. These results will be

related to results obtained from this investigation, and interpreted
in a later discussion, in the light of the results of BANGHAM and
DAWSON (1962), in regard to activation of enzymes with an alter-
ation of change of the charge on the membrane, and especially to
water soluble phospholipase D described by LONG et al (1967,a,b),
and in relation to the LUCY (1964) model of a plasma membrane, in
which water-filled pores, lined with phospholipid, occur.

The function of the sensitive hair of Dionaea, and the structure of membranes.

The functioning of the sensitive hair of Dionaea concerns
essentially the functioning of membranes. If the formation of an
action potential in Dionaea is compared with stretch receptor action
in animals, then the theory that simple distortion of membrane area occurs
can be applied. The understanding of the chemical structure, and
the ultrastructure of membranes, especially of the plasma membrane,
is essential to the interpretation of the functioning of the sensi-
tive hair of Dionaea.

The effects of the action potential from the stimulation of the
sensitive hair, are in the cells of the inner epidermis, and the
effects here are probably due to a breakdown of permeability of the
plasma membrane (STUHLMAN, 1950).

It is for these reasons that the following account of membrane
chemistry and ultrastructure is given.

The structure of membranes.

1. Chemical structure.

2. Ultrastructure.

1. Chemical structure.

The chemistry of membranes has been amply reviewed by COOK (1968). The methods of understanding this chemistry are as follows:-

1) Electrophoretic studies on the cell surface.

2) Chemical analyses by:-

a) Isolating the membranes by various methods.

b) Obtaining their gross composition.

c) Using various methods to determine the carbohydrate components present.

3) Studies of the biosynthesis of membranes.

1) Electrophoretic studies.

The classical model of the plasma membrane as a lipid sandwiched between protein (DANIELLI and DAUSON, 1935) is incomplete without considering the presence of carbohydrate complexes. Electrophoresis together with studies on the chemistry of blood-group substances, has done much to demonstrate the role of carbohydrate substances.

Using enzymatic digestion of cells such as erythrocytes, coupled with electrophoresis and paper chromatography, COOK, HEARD and SEAMEN (1960) followed by other workers, have been able to

demonstrate that much of the negative charge on the outside of certain cells, is due to sialic acid, the role of which has yet to be fully elucidated.

R$_3$NH ... H ... O ... COOH

OH

OH

CH$_2$OH ... H ... OR$_1$

H ... H

OR$_2$
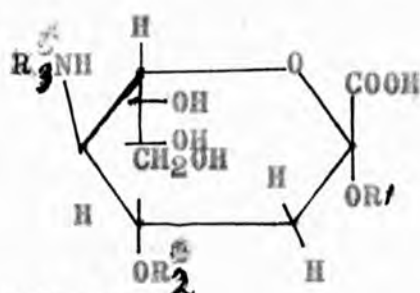
SIALIC ACID

2) Chemical analyses.

a) Isolating the membranes.

Plasma membranes of some cells such as erythrocytes can be prepared as "ghosts" by freezing and thawing, by lytic agents or by the use of media at pH9.

Tissues can also be ruptured by gentle mechanical means, by ultrasonic disintegration, or in a French pressure cell, when the plasma membrane disintegrates into small fragments which sediment. Modifications of the sucrose-flotation technique with centrifugation has been used by EMMELOT et al (1964) to yield plasma membranes in sufficiently pure form for detailed chemical and enzymatic assays, as well as for electron microscopy.

b) Gross composition of membranes.

The lipid content of erythrocyte and liver ghosts is about the same, and to not vary much from animal species to species. There is less lipid present then protein. Phosphatides amount to 50 - 60 per cent of total lipids of "ghosts," and cholesterol accounts for 70 - 90 per cent of the remaining lipids. Protein is

associated with lipids in complexes, and is difficult to study.

c) Carbohydrate components of membranes.

As the dominant factors in determining serological specificity are carbohydrate in nature, it may be assumed that similar chemical moieties are an important feature of cell membranes. Thus GASIC and BERWICK (1963) described how the Hale stain can be used to demonstrate sialomucin under the electron microscope. Various workers have shown that sialic acid effects the functioning of the permeability of membranes. The carbohydrate portion usually contains sialic acid in addition to hexoses like galactose and amine sugars.

3) Studies of the biosynthesis of membranes.

This type of research aims at labelling components of the membrane so as to ascertain the order of assembly of lipid, protein and carbohydrate into membranes. Such research has been carried out with the developing rat hepatocyte by DALLNER, SIEKEVITZ and PALADE (1966a,b). Results have indicated that the new membrane is synthesised in the rough endoplasmic reticulum, and is transferred to the smooth ER (1966a). Further, that the constitutive enzymes of microsomal membranes during a period of rapid development in rat hepatocytes, appear at different times, increase at different rates, and seem to be synthesised in the rough endoplasmic reticulum.

2. Ultrastructure of membranes, especially of the plasma membrane.

In 1962 it was generally thought that the basic structure of the

plasma membrane was essentially understood, and it had been under-
stood for over 20 years (DANIELLI, 1962). This model of DANIELLI
was termed a "unit-membrane" by ROBERTSON (1960) and consisted of a
double layer of lipid molecules with the hydrophobic portions
inwards, the hydrophylic polar groups pointing outwards to form a
"bimolecular leaflet" of phospholipid. DANIELLI suggested that it
was stabilized by adsorbed protein on either side, and that perme-
ability was controlled by protein-lined pores. The evidence for
this model was based on several techniques, such as the behaviour
of lipid monolayers on a Langmuir trough, X-ray diffraction and
ploarization studies, the surface charge, electrical conductance
and capacitance, as detailed by DAVSON and DANIELLI (1943).

Electron microscope pictures supported the classical model.
STOECKENIUS (1962a) made a series of studies on preparations of
lipids from brain tissue which were fixed with osmium tetroxide,
embedded and sectioned; he concluded that the three-layered struc-
ture represents a section through a bimolecular leaflet of lipid
in which the osmium has been deposited at the outer, polar groups
to give the typical triple-layered appearance. Plasma membranes
are typically 70 to 90 $\overset{o}{A}$ thick, and take up stain only to a depth
of 20 - 30 $\overset{o}{A}$ on either side, leaving a clear central region 20 -
30 $\overset{o}{A}$ thick.

Since 1962 the following types of investigations have illus-
trated the need to modify the Danielli model:-

1) The analysis of lipid-water systems by X-ray scattering analysis.

2) The electron microscopy of isolated lipids by the negative-staining technique.

3) The electron microscopy of thin sections of membranes at high magnifications.

4) Freeze-etched studies.

5) Depolymerizing membranes with bile salts, abstracting certain constituents, and then re-polymerizing.

1) X-ray scattering analysis of lipids.

LUZATTI and HUSSON (1962) published the results of their study by X-ray scattering analysis of lipid-water systems, and concluded that at certain temperatures and concentrations the bimolecular leaflet structure exists, but at 37°C and at a low water concentration the surface structure was consistent with lipid lined pores, arranged in an hexagonal pattern. STOECKENIUS (1962b) fixed, embedded, and sectioned a sample of the same preparation of phospholipids under the conditions in which the hexagonal structure appeared to exist; the image corresponded to the interpretation of the X-ray data.

2) The electron microscopy of isolated lipids by the negative-staining technique.

Preparations of lipid-membranes such as lecithin or a mixture of lecithin plus cholesterol, or of intact membranes have

been made since 1962, using the potassium phosphotungstate technique of negative staining. Papers published by FERNANDEZ-MORAN (1962), ELBERS et al (1965), BANGHAM and HORNE (1964), LUCY and GLAUERT (1964), GLAUERT (1965), GLAUERT and LUCY (1967), GLAUERT (1968) and ROBERTSON (1964) all support the micellar model for the lipids of membranes proposed by LUCY (1964). This model suggests that much of the membrane may be in the form of a bimolecular leaflet, with globular protein molecules incorporated into the plane of the lipid layer in communication.

3) The electron microscopy of sections.

To observe the molecular structure of membranes after chemical fixation and sectioning, it is necessary to have sections thinner than 200 Å, otherwise the superpositioning of detail would render impossible the visibility of sub-units.

SJOSTRAND (1963a,b) and SJOSTRAND and ELFVIN (1964) have obtained electron micrographs of certain membranes chemically fixed and later sectioned. These have a globular substructure, of particles 10 Å thick and 45 to 50 Å apart, agreeing with the negatively stained preparations of LUCY and GLAUERT (1964). Such structures are infrequent in plasma membranes, but BENEDETTI and EMMELOT (1966) found that a transition from a lamella to a globular arrangement of plasma membrane elements can be induced by incubation of the membrane at 0°C with citric acid or EDTA.

GLAUERT (1968) noted that the membrane is punctured by

small, aqueous pores with radii of about 4 $\overset{o}{A}$, through the centre
of micelles of lipid, so that no specialized regions like those
postulated by STEIN and DANIELLI (1956) are required to account for
the passage of small water-soluble molecules. The size of the
pores in the model is consistent with the experimental values ob-
tained in a number of investigations, including studies on red blood
cells (PAGANELLI and SOLOMON, 1957-8).

BLASIE et al (1965) observed ordered arrays of particles
about 40 $\overset{o}{A}$ in diameter within the membranes of the outer segments
of retinal receptors examined by electron microscopy of negatively-
stained preparations.

The use of negative-staining of the surfaces of membranes has
revealed regular hexagonal patterns on the surfaces of intestinal
cells, 40 to 60 $\overset{o}{A}$ in diameter and possibly associated with invertase
and maltase activity (ODA and SEKI, 1966), on the liver plasma
membrane 50 to 90 $\overset{o}{A}$ in diameter (BENEDETTI et al, 1965), and on
bacterial surface membranes (FISCHMAN et al, 1967). These probably
represent complexes of protein or glycoprotein on membrane surfaces.

4) Freeze-etching.

BRANTON (1966) has found that frozen membranes fracture
along their inner, hydrophobic surfaces to reveal varying numbers
of particles 85 $\overset{o}{A}$ in diameter. The particles are thought to
represent regions in which the membrane components have assumed
globular or micellar configurations, whilst the smooth regions

between the particles would represent regions in which the membrane components exist as an extended bilayer. These results are supported by the results of MOOR and MUHLETHALER (1963), with yeast; and fits in with the model proposed by LUCY (1964).

5) Depolymerization with bile salts followed by repolymerization.

GREEN et al (1967) have depolymerized membranes with bile salts, and then repolymerised, in some experiments, after removing one or more component. They then either negatively stained, or fixed in glutaraldehyde and sectioned, and examined the preparations by electron microscopy.

They found that without phospholipids, a three-dimensional unit, and not a membrane was formed, and that enzymatic activities and electron transport cannot be fully demonstrated under such conditions. The presence of phospholipids resulted in a continuum one macromolecule thick that can be identified with a membrane, and had full enzymatic and electron transport capacity.

Membrane structure from studies of electro-osmosis.

FENSOM and WANLESS (1966) have studied the number of water molecules per ion which were moved electro-osmotically through cells of Nitella translucens and N. flexilis They calculated that 10^8 to 10^9 pore sites per cm^2 exist on the surface membrane for Na^+ or K^+ ion transport, and stated that their results agree with the results of freeze-etching studies of MOOR and MUHLETHALER (1963)

for the plasmalemma of yeast and root tip cells, which show 10^9 to 10^{11} hexagonal units* per sq. cm. Each hexagonal unit may contain a pore** of up to 8 or even 12 Å in diameter, or might be the site of pinocytic activity as suggested by MACROBBIE (1964).

Membrane permeability and phospholipase action.

As a result of recent knowledge, LUCY (1964) proposed that small aqueous pores with radii of about 4 Å and lined with phospholipid, occur in a DANIELLI-DAVSON model plasma membrane.

The widespread occurrance in plants (and animals) of phospholipases (ANSELL and HAWTHORNE, 1964), and the activation of certain phospholipases by an alteration of the charge on the membrane (DAWSON and BANGHAM, 1961) as would occur from an action potential, suggests a correlation between membrane permeability and phospholipase action, especially where an action potential is evoked. For this reason, the following review of phospholipids and phospholipase activity is given.

Phospholipids and phospholipase action.

Apart from glycolipids, sulpholipids and sterols as types of lipids in plant membranes in significant quantities, phospholipids account for about 50 per cent of the total lipids of certain plants according to WHEELDON (1960). The most abundant phospholipid is lecithin, which has the basic structure:-

* 'Hexagonal units' is used in the sense of "granules in hexagonal array", and
** 'Pore' in the sense of 'space in the hexagonal array'.

$$H$$
$$H - C - R1$$
$$H - C - R2$$
$$H - C - H$$
$$O$$
$$O = P - O - X$$
$$O^-$$
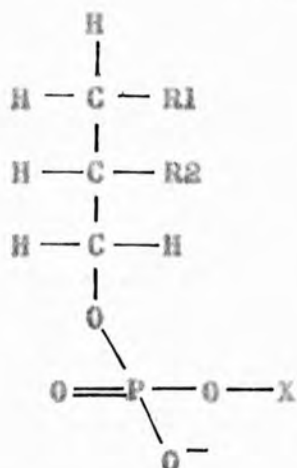
with lecithin (or phosphatidyl choline), R1 and R2 are long-chained
fatty acids, and X is choline. If R2 is substituted by hydrogen,
we have lysolecithin. Other common plant phospholipids are phos-
phatidyl ethanolamine where X consists of ethanolamine instead of
choline, phosphatidyl glycerol where X is substituted by glycerol,
and phosphatidyl inositol where X is substituted by the sugar
inositol.

Lecithin is attacked by phospholipases A, B, C and D at the
following positions respectively to give the hydrolysed product:-

BONDS ATTACKED BY PHOSPHOLIPASES A, B, C, and D

(After ANSELL and HAWTHORNE, 1964.)

$$R2 . C . R1$$
$$H . C . R2$$
$$H . C . H$$
$$O$$
$$O = P - O - X$$
$$O^-$$

B

A

C

D
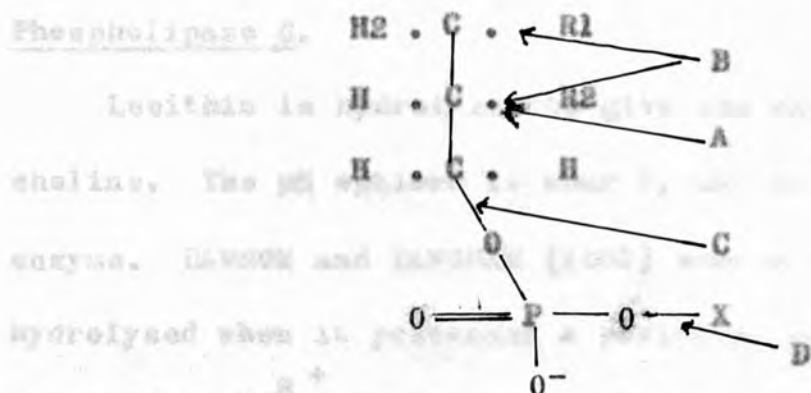
LECITHIN

Both KATES (1960) and ANSELL and HAWTHORNE (1964) have

reviewed the lipolytic enzymes.

Phospholipase A.

This enzyme occurs in pancreas, liver, kidney and other animal tissues, and in a variety of plant tissue as well. The product of its action on lecithin is to produce lysolecithin. It may be a mixture of lecithinase A which is inhibited by Ca^{++} ions, and lyso-lecithinase. Under certain circumstances Ca^{++} ions are necessary, and seem to be involved in binding the substrate to the enzyme. Phospholipase A attacks unsaturated lecithins, but not lysolicithin.

Phospholipase B.

This removes both fatty acids from a phospholipid, and may consist of two separate enzymes, phospholipase A and lyso-phospholipase. This enzyme has been found in certain plant and animal tissues. The enzyme appears to be bound to particles in many instances. Calcium ions do not activate and may inhibit this enzyme.

Phospholipase C.

Lecithin is hydrolysed to give the diglyceride plus phosphoryl-choline. The pH optimum is near 7, and Ca^{2+} ions activate the enzyme. DAWSON and BANGHAM (1961) showed that lecithin was only hydrolysed when it possessed a positive -potential. This could be imparted by Ca^{2+} or long-chain bases. Long-chain acids and $Fe(CN_6)^{3-}$ were inhibitory.

Phospholipase D.

This enzyme has only been found so far in plant tissues.
Phospholipase D, according to ANSELL and HAWTHORNE (1964), hydro-
lyses saturated and unsaturated L- 3- lecithins to give a diglyc-
eride plus phosphorcholine and, to a lesser extent hydrolyses
phosphatidylethanolamine and phosphatidylserine. DAVIDSON and LONG
(1958) prepared a soluble form of the Savoy cabbage enzyme, which
they believed came from the cytoplasmic fraction. This enzyme was
activated by calcium ions, by ether or aliphatic esters or ketones.
The optimum pH was 5 to 6. KATES (1956) and WEISS et al (1959)
found that anionic detergents and phosphatidylinositol activated the
cabbage enzyme, whilst cationic detergents inhibited. ANSELL and
HAWTHORNE (1964) suggested that these substances probably acted as
DAWSON (1965) and DAWSON and HEMINGTON (1967) suggested, by affecting
the ζ -potential at the lipid-enzyme interface, and that the calcium
ions may activate by removing excess phosphatidic acid which would
itself give too high a negative potential.

DAWSON (1967) stated that recent evidence indicated that the
phospholipase A of snake venom and pancreas can split lipoproteins
much more rapidly than phospholipids, and this is sometimes true for
phospholipase C. DAWSON (1967) continued that for any given tissue
phospholipase, its specificity for individual substrates and its
rate of attack in vivo will depend on the way in which the
phospholipids are organized and arranged in lipoproteins, constituting

$$CH_2 \cdot O \cdot CO \cdot R$$

$$HO \cdot CH \qquad O^-$$

$$CH_2 \cdot O \cdot PO \cdot O \cdot CH_2 \cdot CH_2 \cdot N(CH_3)_3 \qquad + EH$$

Lysolecithin   enzyme

$$CH_2 \cdot O \cdot CO \cdot R$$

$$HO \cdot CH \qquad O^-$$

$$CH_2 \cdot O \cdot PO \cdot E \qquad + HO \cdot CH_2 \cdot CH_2 N(CH_3)_3$$

Intermediate   + choline

$$EH$$

$$CH_2 \cdot O \cdot CO \cdot R$$

$$O \cdot CH$$

$$O^- \cdot OP$$

$$O \cdot CH_2$$

$$H_2O$$

$$EH$$

$$CH_2 \cdot O \cdot CO \cdot R$$

$$HO \cdot CH$$

$$CH_2 OPO_3 H^-$$
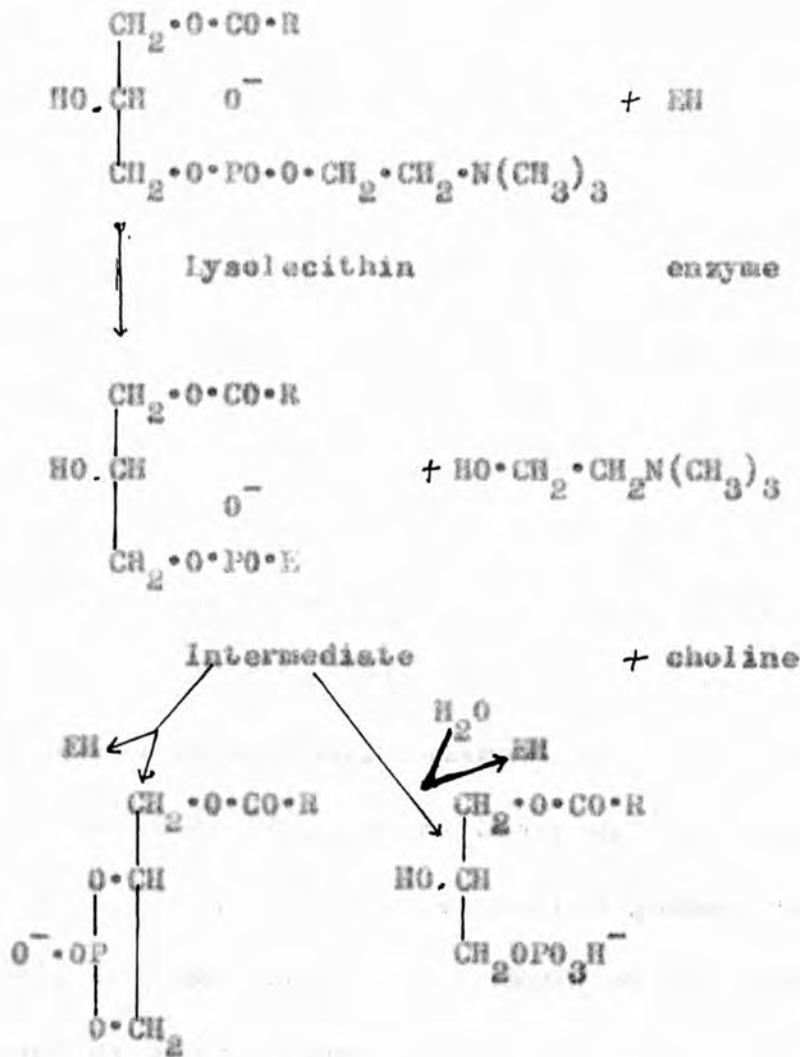
SUBSTANCE A   SUBSTANCE B

(BOTH 'A' & 'B' ARE FORMS OF LYSOPHOSHATIDIC ACID).

These two substances A and B differed greatly in their solubil-
ities, and R f values in chromatography. The relevence of this to
this investigation, is that the enzyme activated by stimulating the
mechanosensory hair has been identified as being a water soluble
phospholipid, with similar properties to LONG's (et al) 'B' form
of lysophosphatidic acid.

The role of calcium in membrane permeability changes and movement in animals and plants.

Because of the similarity in function between the sensory hair of <u>Dionaea</u>, of excitable animal cells such as the Pacinian cor- puscle, and of the pea tendril, below are listed some of the methods in which calcium can act to affect permeability changes associated with receptor mechanisms.

A. <u>Animals</u>.

The action potential from a stimulated nerve causes the release of _previously_ chelated calcium ions in a muscle, and this calcium is the activating ion in muscular contraction. This concept was presented by JÖBSIS (1967), and is based on the pioneering work of HEILBRUNN and WIERCINSKI (1947), of BOZLER (1952), of BIANCHI and SHANES (1959), and more recently of BROWN et al (1963). The kinetic model proposed by JÖBSIS is:-

$$A + C \; \rightleftharpoons \; AC + ATP \; \rightleftharpoons \; A \; ADP + C + Pi\uparrow \; \rightleftharpoons \; A + ADP$$
$$C$$
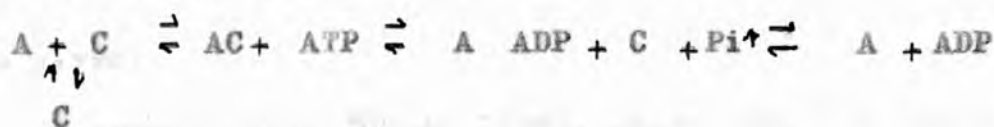
where A = actin-myosin (double protein complex),
    C = the Ca^{2+} activator,
ADP and ATP = adenosine di- and tri-phosphate respectively,
    Pi = phosphate.

The calcium ions act in two ways:

1) By forming a complex with the actin-myosin,

2) by activating the ATP-ase.

JÖBSIS stated that at the risk of oversimplification, as

a result of his experiments it may be conjectured that during the action potential a number of "holes" are created in the membranes of the reticulum and Ca^{2+} diffuses into the sarcoplasm proper.

To summarise clearly the function of calcium, JOBSIS (1967) noted that an action potential, as from a stimulated sensory receptor causes a release of chelated Ca^{2+} ions in cells on the pathway of conduction of the nerve impulse. The protein layers of membranes from widely different sources reviewed by DUNCAN (1967) are considered to have a contractile component, operated by an ATP ase mechanism, and by Ca^{2+} ions. According to DUNCAN (1967) this contractile protein component occurs in such diverse membranes as the pellicle/Amoeba, the erythrocyte membrane, the animal sense receptor and the mitochondrial membrane. Such a mechanism is so general, that the kinetic model for the action of Ca^{2+} released by an action potential on muscle (actin-myosin contractile protein), has been given.

In summarising, DUNCAN (1967, pages 189, 190) indicated that the receptor potential from the sense organ, leads to a propagated action potential in the line of conduction of the stimulus by 1) removing Ca^{2+} ions from their binding sites across pores in the membrane, hence permitting sodium entry, and 2) release of the chelated Ca^{2+} ions, contracting the plasma membrane, and increasing permeability, thus propagating the action potential.

B. Plants.

The following aspects of calcium metabolism are relevant to this study:-

a) The hinge cells of the sensitive hair of Dionaea, which are capable of creating an action potential every five minutes if repeatedly stimulated (JACOBSON, 1965), ~~might be expected to~~ contain numerous mitochondria. FLORELL (1956) has shown that calcium enhances the formation of mitochondria, which are considered to play an important role in ion accumulation.

b) The occurrence of action potentials in plants probably causes calcium to be removed from pores, and an ATP-ase contractile protein activated.

c) Certain phospholipases such as phospholipase B, are activated by calcium ions (DAVIDSON and LONG, 1958). Evidence is produced elsewhere in this dissertation that a permease in membranes of certain organisms, is a phospholipase.

The effects of chlor-choline-chloride (CCC) on membrane permeability.

Chlor-choline-chloride was first used in the current investigation, as an analogue of acetyl-choline, at a stage when muscle-contracting substance in the traps of Dionaea was being studied.

TANAKA et al (1966) found that choline kinase activity in plants was greatly increased between the concentrations 10^{-2} M and 10^{-3} M chlor-choline-chloride. Gibberellin A3 at 4×10^{-4} M inhibited this effect. TANAKA et al interpreted the effect of activated choline kinase, as leading to an increased rate of synthesis of lecithin. This could be expected to decrease the permeability of the plasmalemma of affected tissues, especially if the model of LUCY (1964) of a membrane with aqueous filled pores, lined with phospholipid is correct. Experiments and discussion of the effects of CCC and Giberellic acid on Dionaea traps and sensory hairs, will be discussed later.

Evidence of other research workers that a permease may be a phospholipase.

SLEIN and LOGAN (1967) found that a permeaseless mutant of Escherichia coli which produces β- galactosidase constitutively, when treated with the phospholipases of Bacillus cereus, gave results which suggested that permeability changes may be associated with the activity of a phospholipase that specifically degrades phosphatidyl ethanolamine.

After SLEIN's work, OTTOLENGHI (1967) reported on the lysis

of E. coli spheroplasts by phospholipase C from B. cereus. DINGLE
and LUCY (1962) gave two major possible explanations for the effect
of Vitamin A on the permeability of the plasma membrane of erythro-
cyte ghosts, and one of these was that an enzyme may be activated
which attacks the components of the membranes of cells, and releases
the by-products into the cell.

CHAPTER 10

METHODS, RESULTS, AND DISCUSSIONS OF PHYSIOLOGICAL EXPERIMENTS

Method of growing plants for physiological experiments.

Since most of the physiological experiments were carried out during the winter months (October 1967 to February 1968 inclusive), when the hours of actual sunlight were virtually nil, and the days short, to prevent dormancy, plants were grown at a distance of three feet from "Camplex" (a trade name) Mercury Plant Irradiators. These are marketed by G.E.C. The lamps used were HD 71022, with an "Osram" 400 W. MB/U hard glass lamp as the source of light. The day-length was adjusted to 6 hours, and the temperature at 20°C, with little fluctuation.

Plants were grown in earthenware and plastic pots, having a radius of 10 cms, three plants per pot, as shown on Plate 1, page 1B of this thesis. The pots had a depth of 10 cms, and were filled almost to the top with a mixture of 3 parts sterilised peat, and 1 part by volume of silver sand. At planting time, each pot received 10 mls of Sachs solution, and the medium was saturated with de-ionised water, and all waterings subsequently were done with water from the same source.

Sphagnum was planted on the top of the pots, and under these conditions, for the period under review, all plants in all pots thrived.

METHODS

The presence of a muscle-contracting-substance (MCS) in traps of
Dionaea, its purification and assay.

When
If 0.5 g of traps of Dionaea are ground with 0.1 ml of
eserine (1:200) to prevent breakdown of acetylcholine to acetate
plus choline by ACh ase, and the solution made up to 5 ml with
frog-Ringer solution, a MCS present will contract the Rectus ab-
dominis muscle of the frog. There appeared to be differences in
the amount of MCS present according to whether the sensory hairs
of Dionaea were stimulated or not (Table 2, 19.9.67). The presence
of toxic substance(s) in the traps caused the Rectus muscle to be
killed at an early stage. To lessen this toxicity the following
technique was evolved:

DIALYSIS TECHNIQUE

Traps were harvested when newly formed and open. For one
sample, 0.5 g green weight of traps was taken, being certain that
there was no stimulation. Traps for the samples treated and control
were matched approximately, so that there was the same number and
size of traps. After weighing, traps were placed on a watch glass
with cut ends in a few mls of water, and the watch glass placed in
a Petri dish. After 24 hours any water on the watch glass was
pipetted away. Mechanical stimulation was done with a fine sewing
needle, and fixation by pouring on liquid nitrogen, and placing the
traps immediately in a chamber at -20°C. A mortar and pestle

containing 0.2 g of washed sand was brought to -20°C, and taken out
to room temperature, the sample of traps added together with 0.5 mls
of frog-Ringer solution at 0°C. After grinding at or below 0°C,
the mixture was put into dialysis tubing, and kept frozen until all
the samples were at this stage. The sample was then dialysed in
4 mls of frog-Ringer solution at 37°C for 15 minutes, shaken for
15 minutes whilst allowing to cool to room temperature, then the
dialysis tubing with contents was discarded, and the remaining
solution neutralised with $\frac{N}{3}$ and $\frac{N}{6}$ NaOH, and assayed using frog-
Rectus abdominis muscle. The methods used were those published by
PERRY et al (1968). Muscle contractions were magnified by a weighted
lever which recorded on a smoked, rotating drum of a kymograph. Ten
minutes was allowed between each test solution, whilst the test
muscle was washed with oxygenated frog-Ringer solution.

OTHER METHODS OF ASSAY

1. "Synthesis of Acetylcholine by Brain Tissue" method;

2. Purification of extracts by paper chromatography.

1. For details of the "Synthesis of Acetylcholine by Brain
Tissue" method see Appendix Table 4. The method consisted of
grinding traps of <u>Dionaea</u> with 0.9 per cent saline in the presence
of eserine, incubating at 37°C for one hour, adding $\frac{N}{3}$ HCl, boiling,
filtering, neutralising with $\frac{N}{3}$ NaOH, and assaying with frog Rectus
muscle. A control, which was not incubated, was also run. The
difficulty lay in standardising the time of boiling and losses due

to heat, since the muscle contracting substance is easily destroyed.

RESULTS

The results from assays using frog Rectus muscle, are given in the following tables, 2, 3, 4, 5 and 6, which follow in sequence.

Table 2 shows the differences in the amount of MCS in traps (A) not stimulated, (B) hairs mechanically stimulated, and immediately fixed with liquid nitrogen (i.e. 0 minutes after stimulation), and (C) hairs mechanically stimulated, and fixed with liquid nitrogen 10 minutes after stimulation.

In Table 3 the effects of using identical extracts on different muscles was measured, as well as the amount of MCS some hours after stimulation.

In Table 4, an attempt was made to ascertain whether the MCS was located in the sensory hair, as has been shown for the nettle.

In Table 5 the effect on production of the MCS of closure by protein (in this case yoghurt), compared with mechano-sensory hair stimulation was made.

In Table 6, a MCS was located using similar techniques, in the leaf below the trap of Dionaea, and in other plants.

In appendix Table 3, page 277, a typical record made on the drum of a kymograph using the Rectus abdominis muscle of a frog, is shown.

TABLE 2

Assay of muscle contracting substance, measured by contraction
of frog rectus muscle, ~~as a measure of enzyme activity~~.
(When sensory hairs were touched, and traps were dropped immediately into
liquid nitrogen, the term used is under 'B' below, "0 Mins. after stim.")

DATE	CONTRACTION (mm.) A	B	C	RATIO B/A	RATIO C/A	NOTES ON TECHNIQUE
	NOT STIMU-LATED	0 MINS. AFTER STIMU-LATION	10 MINS. AFTER STIMU-LATION			
19.9.67	3.0		22.0		7.3	Simple grinding with frog-Ringer solution, plus eserine. Traps were not left 24 hrs. after harvest, or treated with liquid nitrogen.
26.9.67	4.0		12.0*		3.0	As for "Synthesis of acetylcholine by brain tissue" method. See Appendix Table 1.
17.10.67	28.0		60.0**		2.1	0.5 g traps, chromatography in 9/1 propanol/ water, paper.

THE MEAN AND STANDARD ERROR FOR B/A & C/A
CALCULATED FOR THE DIALYSIS METHOD BELOW.

DATE	A	B	C	B/A	C/A	
22.11.67	7.5		18.0a		2.4	Dialysis method.
	7.5		11.0		1.5	"
19.1.68	8.8		12.0		1.4	"
31.1.68	6.0		9.0		1.5	"
21.2.68	6.0		10.0		2.5	"
14.11.67	3.5	13.0	9.5**	3.7	2.7	"
7.1.68	8.5	11.0	23.5	1.3	2.8	"
27.5.68	3.5	9.0	8.0	2.6	2.2	"
	3.5	13.0	9.5	3.7	2.7	"
	2.0	3.8	3.8	1.9	1.9	"
	2.0	5.5		2.8		"
21.2.68	7.0	25.0		3.6		"
MEAN				2.80	2.16	
S.ERROR				0.33	0.13	

('a' means 'plus eserine', * and ** mean equivalent to 2×10^{-7} & 10^{-7} ppm
acetylcholine, respectively).

TESTS OF SIGNIFICANCE FOR DATA ABSTRACTED FROM PREVIOUS PAGE.

DIALYSED TREATMENTS ONLY.

When C/A is compared with unity, $t = 45.3$ for 9 degrees of freedom. This means that the values found for stimulation after 10 minutes differ very significantly from the values for unstimulated traps.

The values for C/A are significantly less than those for B/A, as expressed by the means and standard errors.

The t value for the bracketed, 'correlated' (by testing on the same muscle) data is:-
t (difference B/A minus C/A) equals 0.39 for 4 degrees of freedom. The lack of significance in this difference can be explained by the single, anomalous value of 1.3 (7.1.68).

The t value for the non-bracketed, 'not correlated' (tested on different muscles) data is:-
t (difference B/A minus C/A) equals 4.15 for 5 degrees of freedom. This is a significant difference at the 5 per cent level.

TABLE 3

Times after stimulation.

A. The effects of identical solutions on different muscles.
   (There are two rectus muscles per frog; contraction was by
   the dialysis technique).

CONTRACTION IN mm.

Date	Traps not stimu-lated	0 minutes after stimu-lation	5 minutes after stimu-lation	10 minutes after stimu-lation	Muscle No.	Notes
21.2.68	7.0	25.0			} 1	Muscles 1 & 2
	7.0		15.0			from
	4.0			10.0 } 2		same
	4.0		15.0			frog.

B. Extraction by dialysis technique.

CONTRACTION IN mm.

Date	Traps not stimu-lated	0 minutes after stimu-lation	10 minutes after stimu-lation	3 hours after stimu-lation
14.11.67	3.5	13.0	9.5	6.5

C. Extraction by dialysis technique.
   (A and B were tested on different muscles.)

CONTRACTION IN mm.

Date	Traps not stimu-lated	10 minutes after stimu-lation	2 hours after stimu-lation	7½ hours after stimu-lation
22.11.67	A. 7.5*	18.0**		
	B. 9.9		6.5	2.0

* Equivalent to 0.25×10^{-7} acetylcholine.

** Equivalent to 10^{-7} acetylcholine.

*** In the case of both B and C, a control set of 10 traps in
each experiment, stimulated in the same way, commenced to open
after 4 hours.

TABLE 4

The location of the site of the muscle contracting substance in the trap of Dionaea.

Date: 26.9.67

Treatment[*]			Contraction (mm.)
Traps 10 minutes after mechanical stimulation of hairs.	(a)	Control	0
	(b)	Test	12
Traps not stimulated.	(a)	Control	2
	(b)	Test	4
64 large sensory hairs	(a)	Control	0
64 large sensory hairs	(b)	Test	0
50 sensory hairs ground with frog Ringer's solution, incubated for 1 hour.			0
Traps with large sensory hairs removed, 10 minutes after mechanical stimulation.	(a)	Control	0
	(b)	Test	11.5
Acetyl choline, 3×10^{-7}			27.5
Acetyl choline, 2×10^{-7}			11.5

[*] The same mass of traps were ground and used throughout. In the "control" the tissue was boiled first to destroy enzymes, and in the "test" the ground traps were first incubated for one hour at 37.0°C. Details of these are given in Appendix Table I.

TABLE 5

To show whether traps closed for 24 hours because of enclosed protein produced more muscle contracting substance than unstimulated traps.

Date: 26.9.67

Experiment 1

Treatment[x]			Contraction (mms)
Traps unstimulated	(a)	Control	2
	(b)	Test	4
Traps 10 minutes after mechanical stimulation	(a)	Control	0
	(b)	Test	12
Traps fed with yoghurt 24 hours before testing	(a)	Control	2
	(b)	Test	3
Yoghurt 1 gram	(a)	Control	0
	(b)	Test	0
Acetyl choline, 2×10^{-7}			11.5

Method used was as for previous Table. Same mass of traps were used for each treatment. Details of method are given in Appendix Table I.

Experiment 2 (Using the standard dialysis technique)

Traps fed with yoghurt 24 hours before testing	3.0
Unstimulated	4.0
Stimulated mechanically - 10 mins. later	7.0

TABLE 6

The occurrence of a substance which contracts frog Rectus muscle
in other parts of Dionaea, and in other plants.

Date	Species	Mass of tissue (live weight) in grams)	Method of extraction	Contraction (mm.)	
				Extract in frog Ringer solution	Control – frog Ringer
31.1.68	Dionaea Leaf below traps	2.0	Chromatography (paper – propanol/water)	6.0	0.0
31.1.68	Vicia faba End 1 cm. of root tip	2.3	As above	5.5	0.0
27.5.68	Chara (Alga). All plant.	5.0	Dialysis method into 10 ml. frog Ringer solution	3.0	0.0
27.3.68	Linum (Flax). Young shoots.	0.5 g	Standard dialysis method	6.0	0.0
27.3.68	Lactuca seeds	1.0 g	As above	3.8	0.0
27.3.68	Hyacinth roots	1.0 g	As above	15.0	0.0

Identification of muscle contracting substance, and the purification
by paper chromatography.

Methods.

Two methods were used for preparing the muscle contracting
substance for this purpose:

1.   The dialysis extract used for comparative studies was evapor-
ated by a stream of air at room temperature, and the concentrate
spotted onto paper.

2.   Non dialysed trap extract was incubated in the same way as for
the dialysed, was filtered through glass wool, and evaporated by a
stream of air also, and spotted onto paper.

In both cases Whatman's No. 1 chromatography paper was used,
and the direction of solvent flow was down the grain.

Two solvents were used:-

1.   For initial purification, propanol/water, 9/1.

2.   Butanol/acetic acid/water, 60/15/25.

In each case, the length of the run was 30 cms, and the time
at 20°C was 10 to 12 hours.

Frog muscle was used to locate the substance which contracted
frog Rectus muscle. With propanol/water this substance was found
consistently in both dialysed extracts. Rf was 0.8, and could be
reliably located under an ultra-violet lamp, as a band of white
fluorescence, below which was a band of blue-green fluorescence.
This was termed stage 1 of purification.

Further purification was carried out by eluting the frog muscle
contracting substance with water, spotting onto Whatman's No. 1
chromatography paper, down with the grain, using butanol/acetic acid/
water as a solvent. Over several runs, the muscle contracting sub-
stance was reliably found at an Rf 0.95, as a zone of blue fluor-
escence. This stage of purification was termed Stage 2.

Purification was carried to Stage 3, using thin layer chrom-
atography cellulose as the medium and propanol/water, 9/1 as the
solvent. There the Rf value for the muscle contracting substance,
using frog Rectus muscle, was found to be 0.46.

Tests were carried out on the muscle contracting substance,
purified to Stage 3. All of the general locating agents referred
to by SMITH (1960), LEDERER and LEDERER (1957), STAHL (1962),
LEDERER (1960) and MERCK (undated) were used, and a method developed
by LONG et al (1967a), was employed as well.

In applying tests, clearest results were obtained by making
a small spot on chromatography paper, washed in each of the two
solvents used to get to Stage 3 of purity.

RESULTS

Use of locating agents to detect muscle contracting substance
purified to Stage 3.

Of all the locating agents employed, positive tests were only
obtained with those indicative of a lipid, phosphate or phospholipid.
In the list below, only the more specific positive tests are given.
Tests for choline as well as for aminoacids, amines, and related
compounds were all negative.

TABLE **6A** (NEXT PAGE).

TABLE 6 A

Reagent	Process	Reaction
Ammon. molybdate, conc. HCl, perchloric acid, acetone	Paper with extract at Stage 3, dipped in reagent, dried, exposed to U.V. light for 30 minutes. (SMITH, 1960)	Blue spot, phosphate present.
Rhodamine G.	Paper with spot placed in 0.02 per cent rhodamine G in water, then observed under U.V.light. (SMITH,1960;LEDERER,1960)	Blue fluorescence. Positive for a phospholipid.
Bichromate of potassium	Paper with spot of unknown, was immersed in an aqueous solution of pot. bichromate 0.1M, 1 hour at 60°C, then washed in water. (LEDERER, 1960)	No reaction. Choline of phospholipids not present.
2',7'- Dichlorofluorescein	The spotted paper was placed under an U.V. lamp for inspection. (STAHL, 1962)	Yellow fluorescence. Lipid present.
Nile blue	Spotted paper was immersed in a 0.01 M solution in 0.01 M sulphuric acid, and washed well in water. (LEDERER, 1960)	A blue colouration. Positive for acids of phospholipids.
Ninhydrin	0.2 per cent in water-saturated butanol. Reagent sprayed on, then heated 10 minutes at 80-100°C. (SMITH, 1960)	Negative. Primary amino groups absent.
Sodium molybdate, hydrazine sulphate, concentrated H_2SO_4	Sprayed on to chromatogram. (LONG et al, 1967a)	Blue colour, on white background, specific for phospholipid; presence of a long, fatty chain seems to be required, plus a phosphate group.

Further identification of the phospholipid considered to be the muscle-contracting substance in Dionaea traps.   Evidence for the MCS being lysophosphatidic acid.

This was done by five methods:-

1.   By examining its general properties.

2.   By discovering that it possessed lytic properties in low concentrations when applied to animal and plant tissues.

3.   By comparison of the Rf value with that of a known phospholipid.

4.   By the use of frog muscle experiments with the substance derived from Dionaea traps, and the synthesised phospholipid, identified on the basis of findings made in methods 1 to 3 inclusive.

5.   By spectrographic analysis:-

A.   Determining the infra-red spectrum.

B.   Determining the U.V. spectrum.

1.   General properties.

A.   Solubility of this phospholipid in water makes it probable that the substance is either phosphatidic acid, or lysophosphatidic acid.

B.   Using frog muscle as a guide, it was found that:-

1)   The substance was unstable at pH 7 to heat for 5 minutes at 70°C.

2)   It was stable in $\frac{N}{3}$ HCl, boiled for 3 minutes, in unpurified plant extracts.

3) It was unstable to boiling in $\frac{N}{3}$ NaOH for three minutes.

4) It was stable on filter paper (probably because of adsorbed water), but if allowed to dry on a watchglass by a stream of cool air, then taken up in frog Ringer solution, the capacity to contract frog muscle was lost.

5) Chromatograms were run in darkness. On one occasion when chromatograms were run in a room where there was bright, indirect sunlight, the muscle contracting properties were lost.

2. Lytic methods.

A. The frog muscle contracting extract from 2 g of stimulated traps, was purified to Stage 2, and dissolved in 5 ml of water. To this was added 6 washed pieces of beetroot, as cubes with sides 1 mm long. A control was set up with distilled water. The beetroot in contact with the muscle contracting extract lysed immediately, the control did not, even after 24 hours.

B. An extract of the same concentration was placed in a watch-glass, and applied to the backs of 10 traps of Dionaea by immersion of the backs only. After five minutes all of these Dionaea traps had closed, but 10 traps in a control experiment, where distilled water was applied, had not closed. In all cases, the traps were intact on the living plant.

C. An extract of the same concentration as in A. and B., but prepared in 0.9 per cent sodium chloride, was made up, and two

drops (approximately 0.1 ml) of human blood added. The blood immediately lysed, but a control experiment where 0.1 ml of human blood was added to 5 ml of 0.9 per cent saline, did not.

3. Information from 1. and 2. indicated lyso-phosphatidic acid as the frog muscle contracting substance. Reference was then made to the papers by LONG et al (1967, a and b).

Since lyso-phosphatidic acid is unstable, and also very expensive, a sample of lysophosphatidic acid was prepared by preparing a sample of phospholipase D from Savoy cabbage (purified to Stage 3 referred to by LONG, 1967a). This was mixed with lysolecithin, and the pure, standard lysophosphatidic acid was then available.

4. Further identification of the MCS, using frog Rectus muscle.

Two approaches were used:

1) Consisted of the preparation of a solution of the MCS by dialysis, as used in the assay of this substance as a measure of enzyme activity. The solution was divided into two equal portions, one of which was incubated with acid phosphatase from wheat germ. This phosphatase was obtained through British Drug Houses Ltd., Poole, U.K., and its activity was specified at 0.25 E.U. per mg (J. Biol. Chem., 1948, 174, 11).

The results of this experiment are listed in Table 7, page 226, and indicate that a ~~free~~ phosphate group is attached

to the MCS. (This emphasises that a group such as choline,
ethanolamine, inositol, or some other substance, is not attached
to the phosphate group.) These results support the finding that
the MCS is lysophosphatidic acid.

2) The second approach consisted of testing the effect of
a pure sample of lysophosphatidic acid prepared by the method of LONG
et al (1967) by the action of phospholipase D on lysolecithin.
Special emphasis was placed on obtaining the insoluble calcium salt
of lysophosphatidic acid, rendering it soluble as described by LONG
et al (1967), and testing its effect on frog Rectus muscle, whilst
dissolved in 0.6 per cent sodium chloride.

The results indicated that lysophosphatidic acid in
very small quantities would contract frog Rectus muscle, and are set
out in Table 8, page 227.

TABLE 7

Effect of acid phosphatase on a purified sample of muscle-contracting substance.

Muscle contracting from 1 g of stimulated traps substance was purified to Stage 2 by chromatography, divided into two equal proportions in a solution of frog Ringer solution. Half solution was incubated with 0.4 mg of acid phosphatase and made up to 2 ml with frog Ringer solution, and dialysed into 3.5 ml of frog Ringer solution at 20°C for 1 hour. In the other case the MCS was treated with 4 mgm of acid phosphatase, which had been boiled before adding the MCS, and dialysed exactly as previously.

Result   (6.3.68)

		Contraction of rectus
Active enzyme	MCS	3.9 mm
Boiled enzyme	MCS	6.3 mm

TABLE 8

Experiment to show whether lysophosphatidic prepared by the method of LONG et al (1967) would contract frog rectus muscle.

The calcium salt of the acid was prepared according to the method of LONG et al (1967), as a precipitate which was washed, and dissolved as also described by LONG et al (1967). Approximately 3 mgm of lysophosphatidic acid was dissolved in 4 mls of 0.6 % saline, and the contractions compared with a control of 0.6 % saline.

Treatment	Contraction of muscle (mms)
lysophosphatidic acid	8.0
saline (control)	3.5
lysophosphatidic acid	6.5
saline (control)	3.0
Lysophosphatidic acid	8.0

SOME CHROMATOGRAPHIC RESULTS.

Comparisons of the Rf values of the MCS with synthesised
lyso-phosphatidic acid.

TABLE 8A

Rf values of muscle contracting substance compared with those
of lysophosphatidic acid.

(The abbreviations MCS and LPT will be used for each substance,
respectively.)

In each case the length of run was 20 cms upward solvent flow,
temperature 20°C, equilibration time 30 minutes.

| Medium | Solvent | Rf value | | Stage of purity of spot of MCS |
		MCS	LPT	
Silicic acid impregnated paper	di-isobutylketone /acetic acid/ water, 40/30/7	0.12	0.10	Stage 3
Ditto	chloroform/ methanol/water, 65/25/4	0.0	0.0	Stage 3
Ditto	chloroform/ methanol, 4/1	0.0	0.0	Stage 3
Whatman's No. 1 (no silicic acid)	propanol/water, 9/1	0.0	0.0	Stage 1

Other chromatographic results.

It is noteworthy that for paper chromatography, spotted with the crude extract and run in the solvent propanol/ water, 9/1, the Rf value was 0.6 to 0.8, but that the MCS purified to Stage 1, the Rf value was 0.

As already stated, the Rf for the MCS purified to Stage 1, and run in butanol/acetic/water on Whatman's No. 1 paper, 60/15/25, was 0.95.

Thin layer was carried out with a number of solvents, but in all cases the MCS was found very difficult to locate with any locating agent. In the case of Shandon's (Shandon Scientific Company Ltd., 65 Pound Lane, London N.W. 10, England) "Silica gel G" and Kieselgur G, it was impossible to elute the MCS once applied, as tested by frog Rectus muscle.

With cellulose thin layer (using Whatman's thin layer chromedia, CC 41), ascending solvent, 30 minutes equilibration, and a 15 cms run at 20°C, at Stage 2 of purity, testing with frog Rectus muscle, the Rf with chloroform/methanol/water, 65/25/4, was 0.2, and with propanol/water, 9/1, it was 0.6.

Difficulties with location were due to the small quantities of the MCS obtainable.

5. Spectrographic analysis.

a) Infra-red spectrum.

The muscle contracting substance from 12.0 g of

Dionaea traps activated by mechanical stimulation, was purified to
Stage 3, and dissolved in chloroform. The transmission spectrum,
compensated by a similar cell of chloroform, is presented in
Appendix Table 4 , page 278 .

This table indicated that to obtain a worthwhile
result, very much more of the muscle contracting substance would
need to be available - probably from 50 g of traps, mechanically
stimulated. Probably it would be necessary to prepare the MCS in
the dark, to avoid breakdown by light.

B.   Determining the ultra-violet spectrum.

This was done with a similar specimen to that used for
determining the infra-red spectrum.

Absorption was noted in the range 200 to 400 mμ, with
a major peak at 290 mμ, and a shoulder peak at 245 mμ.   A Beckman
spectrometer was used.   Due to shortage of a pure specimen of
lysophosphatidic acid, a comparison of absorption spectra remains
to be completed.   A copy of the recording made is shown in Appendix
Table, 5 , page 279 .   The type of U.V. spectrum obtained is
indicative of an unsaturated, fatty acid side chain.

Identification of the phospholipase involved.

Since the evidence was for the MCS being lysophosphatidic acid, with an unsaturated sidechain, reference to the results of LONG (1967 a and b) indicated that phospholipase D was the enzyme responsible, since the MCS was produced following incubation, and not after boiling traps of Dionaea.

Reference to ANSELL and HAWTHORNE (1964), to BLOCH (1960), and to LONG et al (1967a,b), indicate that phospholipase D activity varies greatly in the range of pH within which it can be active from organism to organism, but there apparently is no case recorded where this enzyme is active at a pH as high as pH 8. The enzyme studied by LONG et al (1967) was water soluble, whereas other forms are particulate bound.

In the attached experiment, using the "Dialysis Technique," and frog Rectus muscle, traps of Dionaea were incubated using standard method, and at pH 8.2, using a) Tris buffer, and b) Normal sodium hydroxide applied immediately after fixation with liquid nitrogen and before grinding.

The results set out in the following page 231 indicate that the enzyme involved, considered to be a phospholipase D, is quite active at pH 8.2.

TABLE 9

Effect of incubating crushed Dionaea traps using the standard dialysis technique, but at pH = 8.2.

Date: 4.4.68

Treatment	Contraction of rectus muscle mm.
½ g traps, incubated with Tris buffer added before crushing pH = 8.2	5.8
½ g traps, incubated without Tris buffer, buffer added later, pH = 8.2	5.0
½ g traps, incubated after being brought to pH = 8.2 with N.NaOH.	5.0
½ g traps, incubated according to standard practice, brought to pH = 8 with NaOH, then neutralized.	5.0

Replication of above.

Date: 12.6.68

Stimulated mechanically, 10 minutes later	7.0
Stimulated 10 minutes later, pH 8.2	7.0
Unstimulated	4.5

THE EFFECTS OF APPLYING CHLOR – CHOLINE – CHLORIDE (CCC)
TO TRAPS OF <u>DIONAEA</u>.

METHODS.

RESULTS.

DISCUSSION.

The ...... method of ....
a needle star all the ...., ...
...... were .... winter ...... the ...
but out to direct ....

Treatment with Gibberellic acid was carried out by dipping the
traps for 1 minute into a 1/1000 solution three times, at 15 minute
intervals, and testing the sensitivity of the trap 24 hours after
the first immersion.

The Gibberellic acid was supplied by British Drug House
Chemicals Ltd., Poole, England, according to the specifications
of their Laboratory Chemicals Catalogue, 1958.

Results are set out in the following table, Table 10A, & Table 10B
page 233.

METHODS

Traps which had newly formed on the plant were selected. A random sample of traps was taken, so that the length of the trap varied from 1 to 2 cms; but in every case the length of lamina below the trap, after the trap plus lamina was cut off, was 3 cms.

For each treatment 3 traps were used. These were placed in open phials, 2.2 cms internal diameter, and an internal height of 2.2 cms, volume 8.5 mls. Into these phials was placed 5.0 mls of the appropriate test solution.

In this case the method of stimulating traps was by passing a needle over all six sensory hairs, six times. The traps being treated were positioned in a room near a window facing the sun, but not in direct sunlight.

Treatment with Gibberellic acid was carried out by dipping the traps for 1 minute into a 1/1000 solution three times, at 10 minute intervals, and testing the sensitivity of the trap 35 minutes after the first immersion.

The Gibberellic acid was supplied by British Drug Houses Chemicals Ltd., Poole, England, according to the specifications of their Laboratory Chemicals Catalogue, 1968.

Results are set out in the following table, Table 10A, & Table 10B page 233.

The effects of chlor-choline chloride (or CCC) on the closing mechanism of Dionaea traps.

The following experiment was replicated three times, and the same results obtained each time.

Concentration	Time leaf bases were immersed (hours)	Effect of stimulating sensory hairs after treatment	Effect of dipping traps through 1/1000 Gibberellic acid after stimulation
M/10	24	Traps withered	No effect
M/100	24	Traps inactivated to 12 stimulations	Traps closed after 1 stimulation
M/1000	24	Traps partially inactivated - closed only after 6-12 stimulations	Ditto
M/10,000	24 48 72	Rapid closure after one stimulation	Ditto
Control - distilled water	24 and 72	Ditto	Ditto

TABLE 10B

The following experiment was replicated three times, and the same results were obtained each time.

M/3	4 4	Traps closed after one stimulation	Traps closed after one stimulation
M/3	5½	Traps inactivated to 12 stimulations	Ditto
M/4	5½	Traps closed after 1 stimulation	Ditto
Control - distilled water	5½	Ditto	Ditto

DISCUSSION

The results on the effects of the action potential produced by stimulation of the sensory hairs, can be interpreted in the light of the results of TANAKA et al (1966), who considered that CCC remains in the plant unchanged, and found that at a concentration of 10^{-2}M CCC, there was an increase in activity of choline kinase of 100 per cent; at 10^{-3}M CCC the increased activity was nearly 50 per cent, and at 10^{-4}M CCC there was no increase in kinase activity.

TANAKA et al also found that Gibberellin A3 at 4×10^{-4}M inhibited the enzyme effect, and Gibberellin plus 10^{-2}M CCC produced a normal rate of kinase activity.

DAWSON (1966) stated that in animal tissues a major pathway for the formation of lecithin, is for phosphorylcholine to be converted to cytidine diphosphate choline (or ethanolamine) via the appropriate cytidyltransferase, and then to lecithin via choline-phospho-transferase. MAIZEL, BENSON and TOLBERT (1956) identified phosphorylcholine as an important constituent of plant sap, whilst TANAKA et al (1966) stated that the effect of activated choline kinase by CCC is to increase production of phosphorylcholine, expected to stimulate lipid synthesis.

GLAUERT (1968) considered that the plasma membrane based on Lucy's micellar model, would contain aqueous pores with radii of about 4 $\overset{o}{A}$, lined with phospholipid, so that no specialised regions

lined with protein as postulated by STEIN and DANIELLI (1958) are required to account for the passage of small, water-soluble molecules.

The expected effect of activated choline kinase by CCC could be to block these pores by synthesised lecithin; Gibberellic acid would reduce synthesis of lecithin, whilst phospholipases would be in a state of dynamic equilibrium, depending on such factors as the Zeta potential (BANGHAM and DAWSON, 1962).

Whether or not the actual action potential of the mechano-sensory hairs is decreased or prevented by CCC by the decreased permeability of the hinge cells, or whether an action potential is elicited; whether the permeability of the plasma membrane of the inner epidermis of the trap is decreased by CCC, is not known. Either the permeability of the hinge cells, or that of the cells of the trap, or both, may be decreased, and could be a subject for future research.

DISCUSSION

PHYSIOLOGICAL
EXPERIMENTS

DISCUSSION OF:-   **1. ALL OF THE ACTUAL RESULTS**
OF PHYSIOLOGICAL EXPERIMENTS.

2. THESE RESULTS IN RELATION
TO PERTINENT, WELL ESTABLISHED
FACTS.

3. IN RELATION TO PERTINENT,
WELL ESTABLISHED HYPOTHESES.

DISCUSSION

From the Review of the Literature, such results as those obtained by JACOBSON (1965) indicated that the mechanism of the sensitive hair is to produce an action potential. STUHLMAN (1950) established that trap closure was a result of loss of turgor pressure in the cells of the inner epidermis. The results concerning the MCS concerned the trap as a whole. In the use of CCC, further experiments are needed to determine whether its effects are in the sensory hair, or the trap, or both.

1. The Actual Results.

The histochemical test for Cholinesterase shown on page 58, indicated that this enzyme was not present. This cast doubt on the presence of an acetylcholine transmitter mechanism, as in certain animal nerve-muscle synapses. The Rectus muscle contracting substance (MCS) was not present in large quantities in the sensory hair of Dionaea, (shown in Table 4, page 219c) whereas EMMELIN and FELDBERG (1947) showed that only one nettle hair would cause a marked contraction of Rectus muscle.

Further indication that the transmission mechanism was not that of a nerve-muscle synapse, was the absence of the need for eserine during the incubation of the crushed traps - some enzyme other than acetylcholinesterase was functional.

The kinetics for the production of the MCS, are shown in tables 2 and 3 pages 219a and 219b. The efficiency of the Rectus method of

RELATIVE AMOUNTS OF MUSCLE CONTRACTING SUBSTANCE IN TRAPS OF DIONAEA, AS A MEASURE OF PHOSPHOLIPASE ACTIVITY, USING THE FROG RECTUS MUSCLE ASSAY.

For B/A, mean is 2.8 , S.E. is 0.33.

For C/A, mean is 2.16, S.E. is 0.13

TRAPS STARTED OPENING.

TRAPS NOT TOUCHED A

TRAPS FED PROTEIN

3 SECONDS AFTER HAIRS TOUCHED B

10 MINS. AFTER HAIRS TOUCHED C

3 HOURS AFTER HAIRS TOUCHED

7½ HOURS AFTER HAIRS TOUCHED

50

100

150

200
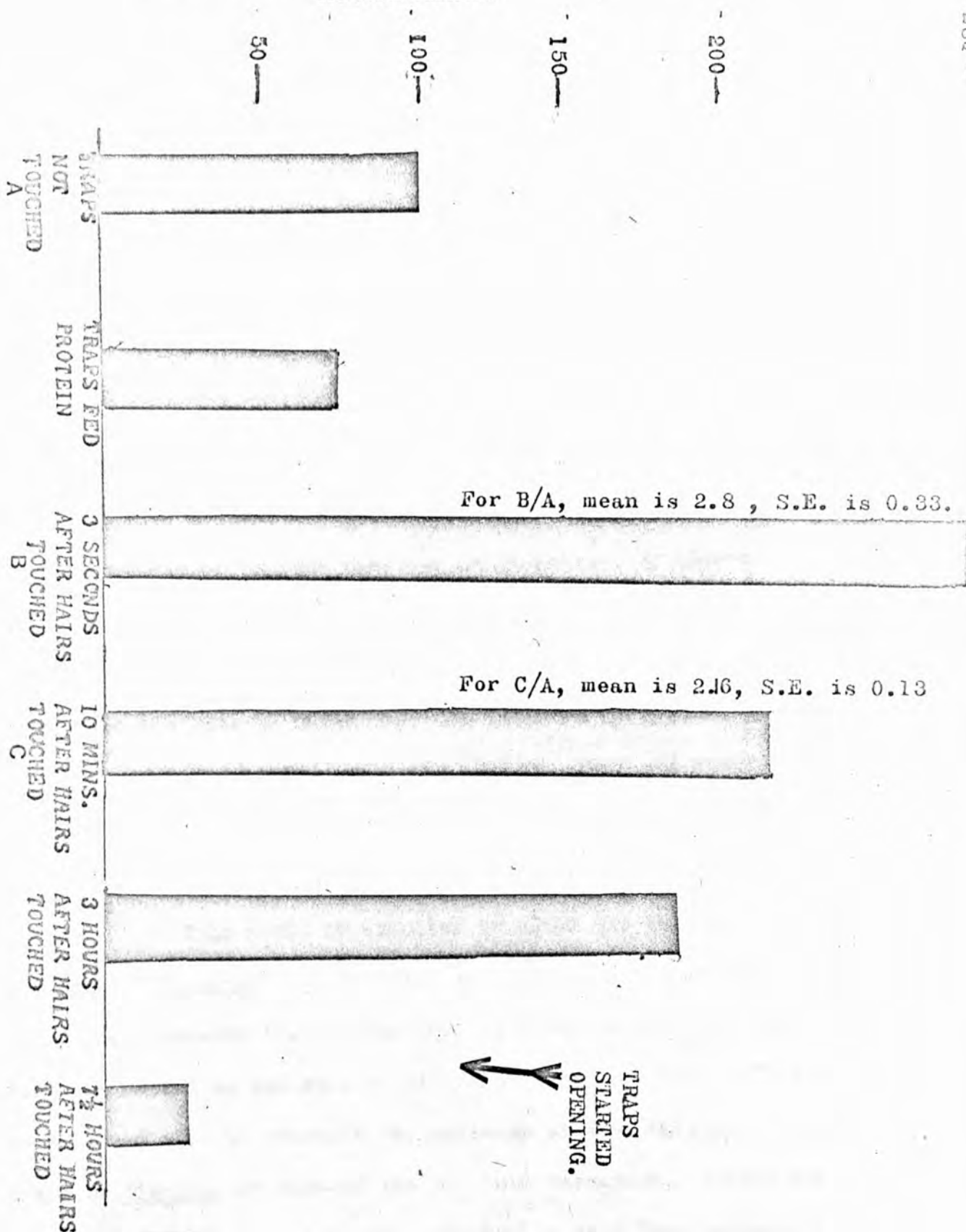
assay, is reflected in the low Standard Error shown in table 2.

Because the MCS was a water soluble phospholipid, and the extensive

tests carried out indicated that it was the 'B' form of

lysophosphatidic acid studied by LONG (1967), there is good

evidence that the effect of touching the sensory hairs of <u>Dionaea,</u>

is to produce an action potential which activates a phospholipase D. A
diagram showing the activity of this enzyme, is shown on page 237 A.

 2. <u>These Results In Relation To Pertinent, Well Established Facts.</u>

  As shown by LONG et al (1967) and DAWSON and HEMINGTON (1966),

certain plants contain a water soluble phospholipase D, which depends

on the presence of Calcium ions and is stimulated by increasing

Ca^{2+} concentration up to 0·039 M at pH 5·8. It has been shown that

the Zeta-potential, or charge on the membrane is often a factor which

determines the rate at which they are attached by phospholipases

(BANGHAM and DAWSON, 1959 and 1962, DAWSON, 1964 and 1965).

  JACOBSON (1965) showed that the action of stimulation of the

sensory hair of <u>Dionaea</u> was to produce an action potential, (as in a

nerve) . This could be expected to alter the Zeta-potential in

the trap of <u>Dionaea,</u> and alter the activity of phospholipase. JÖBSIS

(1967) has reviewed the effects of action potentials, and has indicated

that one result is the release of chelated calcium ions. This may

be expected also to increase the activity of phospholipase D in the

traps of <u>Dionaea,</u> in view of the previous paragraph. DAWSON and

HEMINGTON (1967) have shown that phospholipase D from cabbage will

attack lecithin, and LONG et al (1967) have shown that the same

enzyme will attack lysolecithin.

Since the basic structure of the plasmàlemma, as covered in the literature reviews, has been well established as being lecithin, then the ~~closing mechanism of the sensory hinge~~ action potential can be related to the release of chelated calcium ions, and an alteration of the Zeta-potential, *leading to the closing mechanism.*

Another fact is that CCC at certain concentrations greatly increases the concentration of choline kinase, thus leading to an increase in concentration of phosphorylcholine, which TANAKA et al, (1966) predicted (by the Law of Mass Action) would increase synthesis of lecithin. The results from the effects of CCC and Gibberellic acid traps of Dionaea, correspond to those for the activation of choline kinase, and so lecithin production, found by TANAKA.

DINGLE and LUCY (1962) obtained evidence that the action of Vitamin A in causing lysis of erythrocytes was different from the effects of lysolecithin, because studies of these two lytic agents at different concentrations, indicated different temperature characteristics, and different animal species relationships. This shows that because lysophosphatidic acid may be formed in a cell of the trap of Dionaea, and because the MCS closes the traps of Dionaea, that a lyso-phospholipid is not necessarily the factor involved in membrane permeability increase, even though it is formed in significant amounts when crushed traps are incubated with frog Ringer's solution (which contains Ca^{2+}). Other aspects of the results of DINGLE and LUCY (1962) will be dealt with under the next section, "Hypotheses".

The results from physiological experiments in this dissertation can be interpreted as the lecithin of the plasma lemma being in a state of dynamic equilibrium with enzyme systems, involving synthesis (activated by choline kinase) and breakdown by phospholipase D, (activated by an action potential resulting from mechanical stimulus to the sensory hair).

3.. The Results Obtained In Relation To Pertinent, Well Established Hypotheses.

These will be discussed under two aspects:-

a. Those affecting the central theme of this thesis, which involves membrane permeability.

b. Those affecting the pumping mechanism for restoration of turgor in the trap, and electrical potential in the hinge cells.

a. Hypotheses affecting membrane permeability

It is accepted in this thesis that the detailed appraisal by DUNCAN (1967) of most sensory mechanisms is accurate. In his summary and conclusions he classed the hypotheses for the mechanisms of excitable cells which involved an alteration of the cation permeability properties of their boundary membrane, an ATPase "mechanoenzyme" complex which is capable of undergoing changes in its molecular configuration and/or change distribution as a function of the balance between the ATPase and ATP, and that all sense organs are temperature-sensitive, reflecting their dependence on enzymic maintenance of the membrane. (There is an analogy here with the

transducer mechanisms. Such a mechanism is relected in the model
for the coiling of tendrils, proposed by JAFFE and GALSTON (1968).
Even if such an hypothesis is correct for Dionaea, it does not
conflict with the value of an activated phospholipase D, which
has been shown in Table 3, page 219b, to remain activated for up
to four hours, ensuring the trapping of the prey until protein
digestion occurred of the ensect. As shown in table 5, protein keeps
traps closed, but does not activate phospholipase D. The
"mechanoenzyme" sensory system reviewed by DUNCAN (1967) could act
in the initial closure of the trap, but the effects of such systems
are usually transitory.

Referring again to the results of DINGLE and LUCY (1962), the
hypotheses presented for the action of Vitamin A in affecting
membrane permeability of the erythrocyte, and eventual lysis are:
1. An enzyme system may be activated by alteration of the Zeta-
potential. (From the results of this thesis, the enzyme indicated
is phospholipase D).
2. Vitamin A may alter the structural composition of the membrane.
(This fits in with the LUCY 1964 and 1968 papers and that of
GLAUERT, 1968, in which the hypothesis is presented whereby permeability
is altered by a change from lipids in globular micelles to lipids
in bimolecular leaflets. It is consistent with the changes shown
in the results from electron microscopy section of this thesis,
summarised on page 174.

3. Vitamin A may interfere with reactions essential to the
maintenance of the membrane. (There is an analogy here with the

results obtained for the effects of CCC on the mechanism of closure of the traps of Dionaea).

Another hypothesis is that presented by GLAUERT (1968) that cell membranes contain water filled pores, lined with phospholipid. Such a concept would support the results indicated by this thesis: a water soluble phospholipase D when activated would be more capable of attacking a phospholipid-lined pore, than a membrane coated with protein, (protein-lined pores were proposed by DANIELLI, 1943).

b. The Results In Relation To Hypotheses Effecting The Pumping Mechanisms.

1. The sensory hinge of the trigger hair.

JACOBSON (1965) found that the time for restoration of the potential of the sensory hair, was 5 minutes. This indicates two things:-

a. That the permeability of the membranes returns to the "unstimulated" condition within 5 minutes.

b. That there is an efficient ion pump. This would be indicated, according to HEINZ (1967) by an abundance of mitrochondria located near to the membranes of actively transporting cells. (This is expressed in the results from microscopy, Tables 1A to 1G, pages 154 to 160), and according to CHANCE (1963) and to LIEBERMAN et al (1965), by calcium accumulation in mitochondria associated with insoluble precipitates of calcium salts (as shown in EM photographs of hinge cells).

2. The trap as a whole.

DARWIN (1875) found that following mechanical stimulation
alone, traps took longer than 24 hours to open. Under the
conditions of experiment for this thesis, traps took 4 hours
before they commenced to open, and under all circumstances of
mechanical stimulation, all traps were open within 24 hours.
Probably restoration of full turgor in the traps is a synonymous
condition to that of restoration of the potential of the trigger
hair hinge, but table 3 page 219B showed that the opening of the traps
could be correlated with the return to normalcy of the activity of
phospholipase. The rapid return to the normal condition of the
trigger hair hinge shows that the activity of a phospholipase in
this region (not yet shown by experiment to be present, as has been
done for the trap as a whole), has been restored within 5 minutes.
This could be explained partly by removal of calcium ions by the
numerous mitochondria in the hinge cells, as shown by LIEBERMAN et al
(1965). The result of removal of calcium ions would be a lessening
in activity of phopholipase D.

In both the hinge cells, and the epidermal cells of the trap
the actual pumping mechanism can be explained on the Mitchell
Hypothesis, first promulgated by MITCHELL (1961).

The Mitchell Hypothesis.

Mitchell (1961) proposed the phosphorylation theory so that the

respiratory chain is organised in the membrane in such a way that

protons derived from oxidation of the substrate, are delivered to the outside of the mitochondrial membrane, and hydroxyl ions (from reduction of oxygen), to the inside compartment. MITCHELL (1961) proposed that ATP formation occurred by the reversible action of an ATPase located in such a manner so that protons and hydroxyl ions formed during the dehydration of ADP and Pi, are discharged on opposite sides of the membrane, leading to a charge separation. MITCHELL (1961) suggested another important idea. If the electron carriers are orientated so as to extrude protons from the interior of mitochondria during active transport, the effect would lead to cation uptake. This point has been elaborated by REID, MOYLE AND MITCHELL (1966) and MITCHELL (1966) where it is stated that certain cations, especially calcium, cross the membrane more readily than other cations.

The Mitchell Hypothesis has received considerable interest since 1961, but it still is the centre for explaining ion transport, and is the focal point of such reviews of ion transport as those by ROBERTSON (1967) and by LEHNINGER (1964).

LEHNINGER (1964) noted that active swelling is stimulated by respiration, and has a high Q 10, consistent with a chemical or enzymatic process. Calcium ions also cause swelling of mitochondria, and LEHNINGER (1964) suggested that this may be because these ions are a potent uncoupling agent. The latter two points, increased respiration rate and the freeing of calcium ions following an action potential, would explain the swelling of mitochondria already noted in the hinge cells following stimulation.

GENERAL CONCLUSIONS.

GENERAL CONCLUSIONS.

1. From A Study Of Fine Structure of The Hinge of The Sensory Hair.

From the Resume of Results from Fine Structure, pages 173 to 175, the appearance of the fine structure of the hinge of the sensory hair has been summarised. This hinge region was where BENOLKEN & JACOBSON (1967) found that the action potential originated. As shown in plates 13 to 38, pages 69 to 118 inclusive, the fine structure of the hinge cells and that of their medullary cells resembled the fine structure of animal receptors (e.g. the Pacinian corpuscle), and of the nerve-muscle synapse. This similarity was evidenced by the high density of mitochondria, shown in table D page 157 to be 3.13 per 100 square microns, for silver sections (cut at a thickness of 600-800 Å). In the hinge cells and the animal cells just mentioned there were visible large numbers of vesicles. These vesicles are illustrated in EM photographs of the hinge cells, presented from page 52 to 146 inclusive.

The hinge cells of the sensory hair had as well abundant ER. In pages 35 to 37 of the Review of Literature, this was linked with the fine structure of tissues which are engaged in active transport, such as phloem, raphide-forming cells, and salt secreting glands of the mangrove.

In almost all sections of hinge cells viewed, numerous, small plasmodesmata occurred, which ramified through most of the walls of the hinge in all planes. These could be functional in increasing the area of membrane distorted, and hence in increasing sensitivity, because DUNCAN (1967) concluded that the degree of excitation of the Pacinian corpuscle was proportional to the area of membrane distorted.

The Effects of Bending the Hair on Fine Structure.

These are set out in table E, page 158, for sections of hairs represented in plates 12 to 21 (excluding plate 20), pages 67 to 85. A photograph of a section of an unstimulated hinge cell is

exemplified by plate 27 page 97. The fine structure of a bent hinge
cell is typified by the EM photograph shown in plate 48 page 138.
Bending caused: (i) the appearance of myelin forms,

(ii) in three cases out of four, swelling of some
mitochondria.

This represented a similar change to that noted by DANIEL,
DINGLE et al (1966) for rat fibroblast tissue treated with excess
vitamin A alcohol. They interpreted their results in terms of the
LUCY (1964 and 1968) model of membrane structure, in which phospolipids
in the membrane changed from the globular micelle form, to the more
permeable, bimolecular leaflet form.

Other Organelle Counts & Their Movement When The Sensory Hair Was Bent.

Tables 1A and 1B, pages 154 and 155 set out the actual
counts of mitochondria, plastids and Golgi in ten different hairs.
These are analysed in table 1D page 157. The section represented by
plate 20 was not included in the final analysis because it was a gold
section. All other sections were "silver", and had a thickness of 600 to
800 microns.

From table 1D it can be seen that there were 0.29 plastids
per 100 square microns. These were of a proplastid type. These plastids
may not have any special function, if the mechanism of generating an
action potential is comparable with the mechanism considered by
DUNCAN (1967) to exist in the Pacinian corpuscle. In the Pacinian
corpuscle PEASE et al (1957) found only numerous mitochondria and
vesicles.

The number of Golgi in mature hinge cells is low, and they
are absent in most bent hinge cells. DANIEL, DINGLE et al (1966) found
that for rat fibroblast tissue treated with excess vitamin A alcohol,
Golgi distended, and became indistinguishable. As stated in the second
paragraph of this page, DANIEL, DINGLE et al interpreted their results
in terms of the LUCY model, as a change in the orientation of
phospholipids in the membranes of the fibroblast tissue.

Cells immediately above and below the hinge always contained normal chloroplasts, as illustrated in plate 31 page 105. Counts of organelles below the hinge are set out in table 1C page 156, and follow a similar pattern as for the hinge region - relatively few Golgi and plastids, but a high concentration of mitochondria.

In tables 1 E, 1F and 1G pages 158, 159 and 160, an analysis of the movement of mitochondria with bending has been made. In both unbent and bent hairs there was an increased density of mitochondria in the middle of the hinge cells. There appeared to be a movement of mitochondria away from this mid-region with bending, but this did not reach significance. In view of the large error between plants in the Analysis of Variance, page 159, more hairs and more sections per hair need to be analysed, in order to reduce this source of error.

The sensory hair of _Dionaea_ was shown on pages 161-2 to stand considerable abuse by over-bending, and still to be very sensitive 24 hours later. This may indicate the ability of the hinge cells to recover from damage, and requires further investigation.

2. Conclusions from Physiological Experiments.

A.   Acetylcholine appeared to be absent as a chemical transmitter. This was indicated (i) histochemically on page 58, where acetylcholinesterase was looked for, but not found.

(ii) Eserine did not augment the amount of muscle contracting substance (MCS) in crushed, incubated traps of _Dionaea_. An increase in the amount of MCS is expected in crushed, incubated animal synapses, because eserine prevents the breakdown of acetylcholine by acetylcholinesterase. This suggested that a different mechanism operated for the MCS in _Dionaea_ from the mechanism in the nerve-muscle synapse.

(iii) A muscle contracting substance (MCS) was found in crushed traps of <u>Dionaea</u>. This was purified, and identified by means of chromatography as described in pages 220-228. There was only one MCS found, which had strong lytic properties, and in low concentrations closed traps of <u>Dionaea</u> , as described in pages 224-5. The MCS was a water soluble phospholipid, which suggested the 'B' form of lysophophatidic acid described by LONG et al (1967), by the action of phospholipase D on lyselecithin, as set out on page 208.

B. Further identification of this MCS was made by synthesising some 'B' lysophosphatidic acid, as described in page 208, and in detail by LONG et al (1967). Using chromatography paper, and silicic acid treated paper, as well as three different solvents, comparable Rf values were found for the MCS of <u>Dionaea</u> traps, and for 'B' lysophosphatidic acid described by LONG et al. These Rf values have been set out in table 8A, page 227A.

Frog rectus muscle experiments were used for identification purposes. In table 7, page 226, it was shown that the MCS when incubated with acid phosphatase enzyme lost much of its muscle contracting properties. This indicated that there was a free choline group attached to the MCS. In table 8, page 227, it was found that the 'B' lysophosphatidic acid prepared according to the method of LONG et al (1967), contracted frog rectus muscle.

The Infra-red and Ultra-violet spectrums are given in pages 278 and 279 in the appendix of this thesis.

These are consistent with the MCS being lysophosphatidic acid, although shortage of the MCS and of synthesised lysophosphatidic acid prevented further investigation. The instability of the MCS to heat, light and drying noted on pages 223-4, is consistent with the properties of lysophosphatidic acid.

C. The amount of MCS present after touching the large, sensory hairs was measured, as set out in tables 2 and 3, pages 219A and 219B, for different times after stimulation.

Table 5, page 219D showed that protein (yoghurt) fed to traps 24 hours earlier to keep them closed, did not activate a phospholipase. STUHLMAN et al (1949) and STUHLMAN (1950), linked closure of traps with a loss of turgor in the traps, specifically the cells of the inner epidermis of the trap. From tables 2 to 4 this loss of turgor is associated with a phospholipase, probably D, but closure by fed protein involved some other mechanism (table 5).

Table 6, page 219E, showed that a MCS was present in the leaf below the traps, as well as in diverse plants.

D. It was shown that stimulation of the sensory hairs greatly activated this phospholipase D in the traps of Dionaea.

This explained in terms of : (a) release of chelated calcium ions by the action potential as reviewed by JÖBSIS (1967) and the activation by calcium ions of phospholipase D, noted by LONG et al (1967) and DAWSON and HEMINGTON (1967).

(b) the alteration of the membrane potential by the action potential

could be expected to alter the activity of phospho-
lipase D, as shown by DAWSON (1965), and in his
earlier work.

E.   The effects of chlor-choline-chloride or CCC and
the reversal of these effects by gibberellic acid,
on the closing mechanism of Dionaea traps, have been
set out in table 10A, page 233. These results fitted
the concentrations used by TANAKA et al (1966) which
showed that CCC activates choline kinase, and that
gibberellic acid reversed this effect. TANAKA et al
considered that activation of choline kinase would
increase the concentration of phosphorylcholine,
and so lead to increased lecithin synthesis. In the
traps of Dionaea, this could be expected to decrease
the permeability of the plasma membrane of all cells,
and so suppress an action potential from the hair,
and suppress loss of turgor in the traps considered
by STUHLMAN (1950) to cause trap closure.

F.   The concept is proposed that the plasma membrane is in
a state of dynamic equilibrium, being broken down by
phospholipase D, and constructed by an enzyme system
which involves choline kinase.

G.   The hinge cells of the sensory hair recovered quickly
from the effects of stimulus (5 minutes, compared with
from 4 to 24 hours for the trap as a whole). This was
explained in terms of the large concentration of
mitochondria present in the hinge cells (a) absorbing
calcium and other cations, and (b) altering the charge
in these cells, as expalined by the MITCHELL hypothesis,
discussed on pages 243 and 244.

H.   GLAUERT (1968) presented the hypothesis that certain membranes contain water-filled pores, lined with phospholipid. Such a model would support the effects on permeability of an activated phospholipase, and of CCC. This is because a water-soluble phospholipase D described by LONG et al (1967) could fill these pores, and attack the phospholipid more readily.

I.   The basis of present hypotheses for the effects of action potentials on permeability in sensory systems in animals as reviewed by DUNCAN (1967), and as proposed by JAFFE and GALSTON (1968) for the coiling of pea tendrils, is an ATP ase operated contractile protein in the plasma membrane. This still could be the case in the hinge cells and traps of <u>Dionaea</u> following mechanical stimulus and an action potential. However, such effects are usually transitory, and the need for a further method in increasing permeability by activation of a phospholipase extending over several hours, seem logical.

J.   The results from bacterial research, which indicated that a permease could be a phospholipase, gives support to the results for an activated phospholipase D altering permeability in the traps of <u>Dionaea</u>. The results from studies with bacteria were obtained by SLEIN et al (1967) and by OTTOLENCHI (1967).

CHAPTER 13.

SUGGESTED LINES FOR FUTURE RESEARCH.

SUGGESTED LINES FOR FUTURE RESEARCH.

1.   Electron Microscope Studies.

A.   As indicated in page 248, further studies need to be carried
out to determine (a) the movement of mitochondria with bending,
and   (b) the ability of hinge cells which have been
damaged by over-bending to repair this damage, in view of the
continued sensitivity of damaged cells to close traps 24 hours
after being damaged.

B   The ontogeny of the hinge cells requires to be investigated, in
relationship to the threshold of sensitivity of the sensory hair,
and its ability to produce an action potential, and to cause
trap closure.

2.   The Assay of Choline from Phospholipase D Activity.

Phospholipase D has been found by LONG et al (1967) to break
down lysolecithin to produce choline and lysphosphatidic acid.
A form of the latter acid is considered to be the substance
assayed from incubated, crushed traps of Dionaea , using the
frog rectus muscle. The difficulty in using rectus muscle was
that this muscle is easily damage, especially by many substances
in plants.

Phospholipase D activity could be measured using the choline
assay described by LONG et al (1967). In this way, not only
the breakdown of lysolecithin, but also the breakdown of
lecithin, would be measured. The method consists of precipitat-
ing choline, so as to form choline enn-iodide. This was dissolved
in chloroform, and the extinction was read off at 365 mμ.
Standards were run simultaneously, and a linear relationship
between choline concentration and extinction obtained.

3.   The Study of The "Memory" of The Sensory Hair of Dionaea.

JACOBSON (1965) briefly reviewed the evidence for "memory", or the summation over a period of time , up to some hours, of subthreshold stimuli. A similar phenomenon was noted by GRAY (1959) for the Pacinian corpuscle .

DUNCAN (1967) in reviewing memory in animals, described an apparent association between memory and protein production. There is evidence (1) that the antibiotic puromycin prevents protein synthesis by blocking RNA, and (2) that certain injections of RNA can improve memory.

Memory in the Dionaea hair may also be due to accumulated changes in the membrane potential, and in the release of chelated calcium ions causing activation of a phospholipase or other enzyme, until a threshold is reached, and the action potential is discharged. To determine whether or not this latter alternative is possible, then a series of subthreshold stimuli could be given to the sensory hinge of the hair of Dionaea, in quick succession, before there is time for protein synthesis.

- 256 -

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R E F E R E N C E S

The abbreviations used are those given in "Abbreviated Titles
of Biological Journals", compiled by P.C. Williams, on behalf of
THE BIOLOGICAL COUNCIL, c/o Institute of Biology, 41 Queen's Gate,
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Table 1   -273B-

A P P E N D I X

Synthesis of Acetylcholine by Brain Tissue.

(A standard teaching schedule).

Method.

Remove the brains from two rats (killed by a blow on the head). Cut each brain in half and remove blood clots. Take two half brains from different animals as test and the other two half brains as control. Weigh test and control tissues.

Control.

Grind brain tissue with 3 ml 0·9% saline, 1 ml $\frac{N}{3}$ HCl and 0·1 ml eserine (1 : 200). Transfer to a flask, washing with a few mls saline. Boil and filter through cotton wool into a 25 ml measuring cylinder. Make filtrate up to about 10 ml by washing precipitate. Neutralise with $\frac{N}{3}$ NaOH and add saline to give a final dilution of 10 ml extract per gram of tissue.

Assay control for acetylcholine using rectus abdominis muscle of frog.

Test.

Grind brain with 3 ml 0·9% saline and 0·2 ml eserine. Wash into a flask and incubate at 37°C for one hour.

After one hour add $\frac{1}{3}$ ml N.HCl, boil and filter through cotton wool. Neutralise with $\frac{N}{3}$ NaOH and dilute extract further to give

10 ml extract per gram of tissue.

Assay this extract for acetylcholine.

Note.

One guinea pig brain can be used – one half of the brain is then used for the test and the other half for the control.

Appendix Table 2.

A modification of Koelle's method for Cholinesterase

(A standard teaching method).

Acetylthiocholine is hydrolysed by the enzyme. In a medium containing copper, copper-thiocholine is precipitated at the site of the enzyme. On treatment with ammonium sulphide the precipitate is transformed to black copper sulphide.

Medium

|			
$CuSO_4$	2·4 g.	-	to precipitate thiocholine
$MgCl_2$	8·0 g.	-	to activate enzyme
Glycine	3·0 g.		
Maleic acid	14·0 g.	-	to buffer.
1N NaOH	240 ml.		

Hot saturated $NaSO_4$, 1,360 ml. - to prevent diffusion of enzyme or precipitate.

Method.

1. Dissolve 80 mg. acetylthiocholine iodide in a _few_ drops of distilled water in a Coplin jar. Add 40 ml. medium.

2. Incubate frozen sections, or small whole muscles, with the mixture at room temperature for one hour.

3. Wash three times for 2' each in saturated Na_2SO_4, cold and at room temperature.

4. Add about 15 drops yellow ammonium sulphide to a coplin jar of

saturated Na_2SO_4 and leave muscle pieces or sections in this
for at least 5' – covered.

5. Wash briefly in running tap water.

6. Examine under microscope. Cholinesterase activity is indicated
by brown-black deposits.

7. If desired, muscles may be dehydrated, cleared and mounted in
the usual way – great care being taken to remove all traces of
water and to keep preparations as flat as possible. Sections
should be dehydrated, cleared and mounted but may be **counterstained**
if desired before this.

APPENDIX — 3
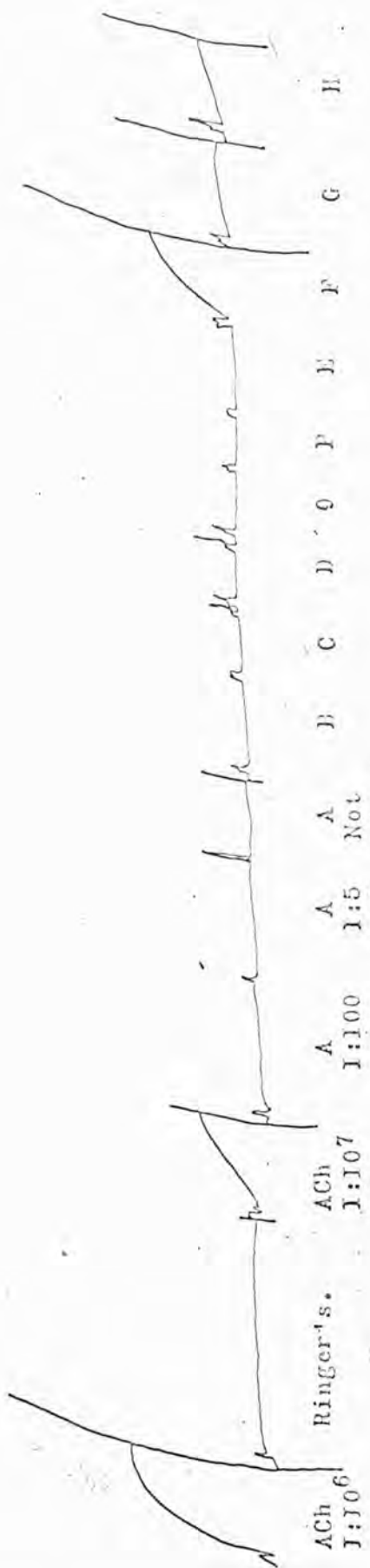
Typical record made
on the drum of a
kymograph, using the
Rectus abdominis
muscle of a frog.
ACh= acetylcholine,
a standard dialysis
technique was used,
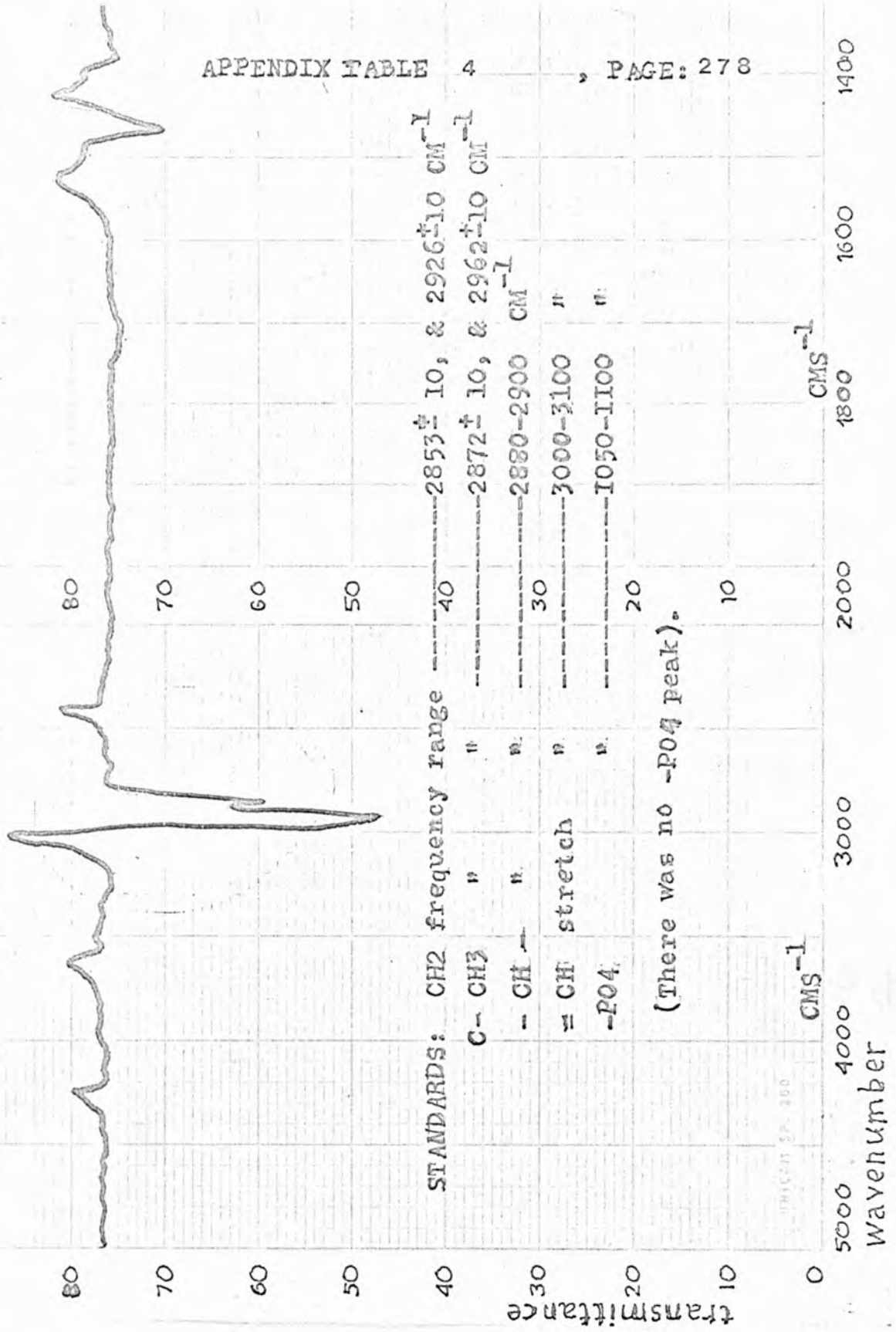stimulation was by
touching sensory
hairs.

G = traps fed protein,

H = unstimulated,

F = traps fixed 10
   minutes after
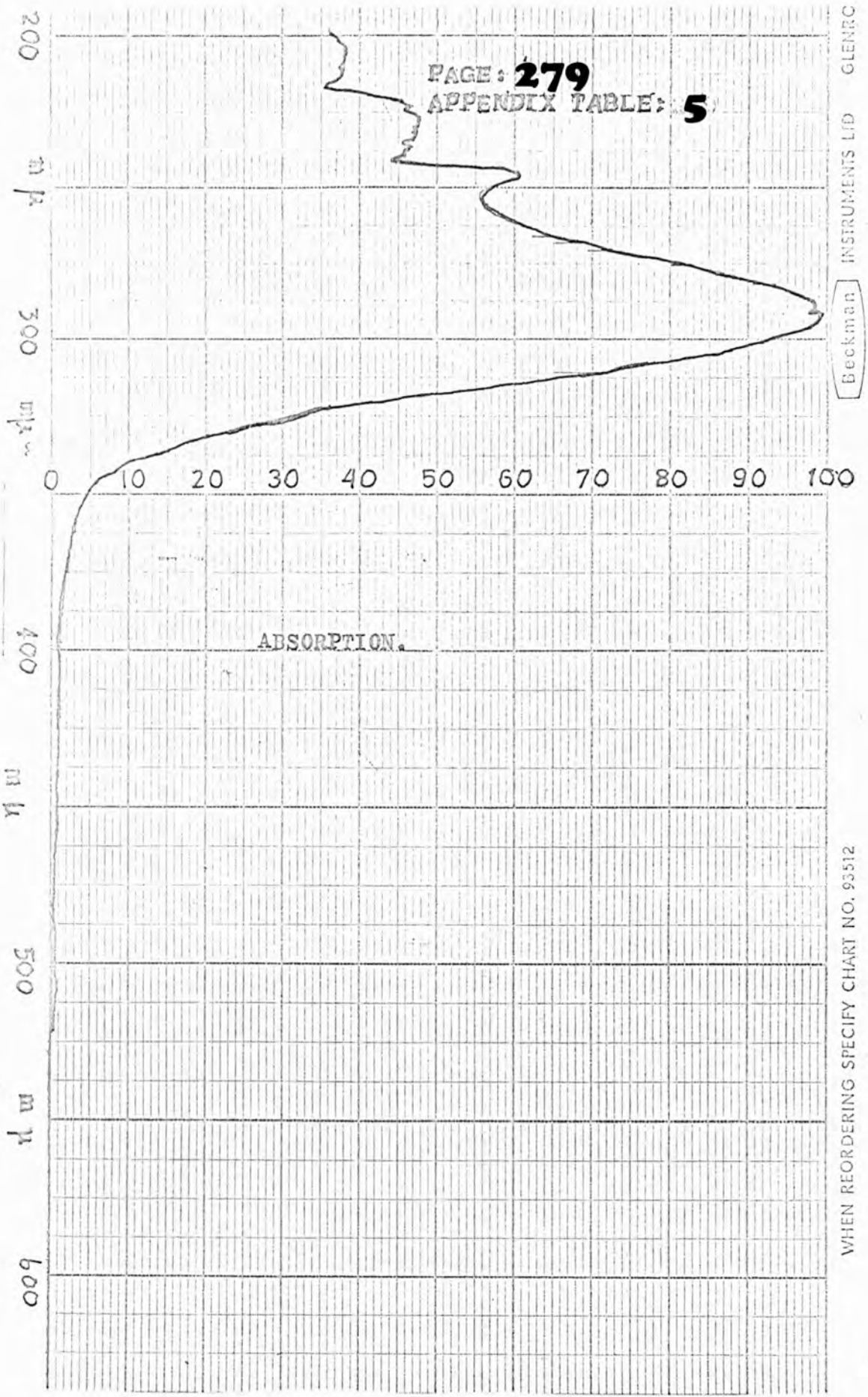   stimulation.

"Not", 1:5 and 1:100
for A refer to traps
boiled before
incubation, and then
diluted; "Not" was
undiluted.

Labels along the record (top to bottom): H, G, F, E, D, C, B, A Not, A 1:5, A 1:100, ACh 1:10⁷, Ringer's., ACh 1:10⁶

INFRARED SPECTROPHOTOMETER   UNICAM SP. 200

MCS DISSOLVED IN CHLOROFORM COMPARED WITH CHLOROFORM CELL. 8.3.1968.

APPENDIX TABLE   4   , PAGE: 278

STANDARDS: CH2 frequency range ——2853 ± 10, & 2926 ± 10 CM⁻¹
            C— CH3   "   ——2872 ± 10, & 2962 ± 10 CM⁻¹
             — CH —   "   ——2880-2900 CM⁻¹
            = CH stretch   "   ——3000-3100   "
            —PO4   "   ——1050-1100   "

(There was no —PO4 peak).

Wavenumber
transmittance

PAGE: **279**
APPENDIX TABLE: **5**

ABSORPTION.

Beckman   INSTRUMENTS LTD   GLENRC

WHEN REORDERING SPECIFY CHART NO. 93512