IN VITRO RECTAL TRANSPORT AND RECTAL ULTRASTRUCTURE IN THE DESERT LOCUST (*SCHISTOCERCA GREGARIA*)

By

H. Barry Irvine

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We accept this thesis as conforming to the required standard

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Department of Zoology

The University of British Columbia
Vancouver 8, Canada

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ABSTRACT

The rectal pad of Schistocerca gregaria consists of a layer of large columnar epithelial cells and a layer of smaller oval-shaped cells. Both layers appear specialized for transport, as judged by the large number of mitochondria and membrane infoldings within the two cell types. The ultrastructure of the columnar epithelium and of the secondary cells is described as it appears under the electron microscope.

The ability of the rectum to transport water and salts was tested in vitro. Unlike the in vivo preparation, the rectum in vitro does not transport potassium and chloride and has only a limited ability to transport sodium and water against a gradient. Dinitrophenol (10^{-3}M.), iodoacetate (10^{-2}M.) and ouabain (10^{-2}M.) abolish water and sodium transport. Potassium cyanide (10^{-2}M.) and ouabain (10^{-3}M.) do not appear to inhibit water or sodium transport. Iodoacetate (10^{-3}M.) inhibits sodium transport but does not affect water transport. The in vitro rectum is dependent upon anaerobic respiration. The results are discussed in terms of a scheme presented for in vivo cellular function. (Phillips, 1965).

The studies of ultrastructure and transport physiology of the locust rectum do not refute the hypothetical schemes presented in this thesis.

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GENERAL INTRODUCTION.

Osmotic and ionic regulation and the physiology of excretion in insects have been reviewed by Shaw and Stobbart (1965). Briefly, the excretory system consists of a number of blind-ended Malpighian tubules, with walls one cell thick, adjoined to the gut at the junction of the midgut and hindgut. These tubules pass a filtrate of the major blood components into the gut and thence to the rectum. Early investigators (e.g. Wigglesworth, 1932) suggested that the rectum reabsorbed water and salts. From measurements of ion concentrations in various parts of the gut of several insects, it was suggested that sodium, potassium, chloride, ammonium, and carbonate ions were actively absorbed in the rectum, although conclusive evidence was lacking (reviewed by Shaw and Stobbart, 1965). Rectal ion absorption was substantiated by Ramsay (1950, 1953), working on mosquito larvae, and by Phillips (1961) on the desert locust, Schistocerca gregaria Forskal. The latter author showed that the locust rectum actively transported sodium, potassium, and chloride from the lumen against electrochemical gradients, independent of solvent flow. A principal function of the rectum in most insects therefore is selective reabsorption of ions secreted by the Malpighian tubules, and the organ is thus of central importance in maintaining osmotic and ionic balance.

Like many terrestrial insects, the desert locust

produces strongly hypertonic excreta (Phillips, 1964). In studying water absorption from the rectum of the locust, Phillips discovered that this hypertonicity was accomplished by water reabsorption from the rectal lumen against an increasing osmotic gradient which could not be accounted for by a hydrostatic pressure gradient, electro-osmosis, or a net simultaneous uptake of solute. The conclusion was that water was being actively moved across the rectal epithelium.*

In many instances, (eg. Curran, 1960; Diamond, 1962) water movement against a gradient has been shown to accompany salt transport by co-diffusion. In Schistocerca, a net solute movement is not apparently necessary for water movement in vivo, although transport and back diffusion of ions are continuously occurring (Phillips, 1964). It is possible that water movement, (17 ul./hr. in the absence of an osmotic gradient) is being driven by this active circulation of ions, thus not involving net salt movement across the rectal wall. Three hypotheses as to how an independent transport of water might be driven by a local salt pump are shown in Fig. 1 (courtesy J. E. Phillips).

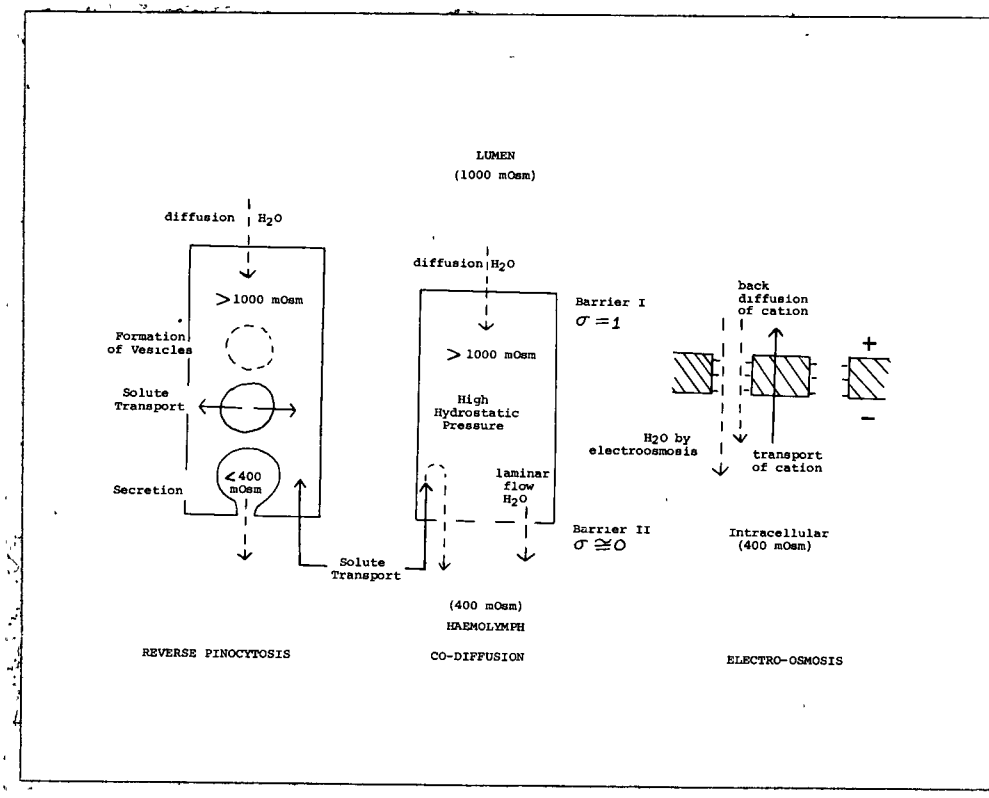
The first hypothesis involves reverse pinocytosis. If the cell interior were made very hypertonic by an ion

*Phillips defines active transport of water as a net movement of water against an osmotic and hydrostatic pressure gradient, which results in an increase in the osmotic pressure of the solution from which the absorption takes place, and resulting in an increase in the osmotic pressure gradient across the membrane separating the two solutions.

Fig. I.

Three schemes which might give rise to active transport of water, in absence of net salt transport. (Courtesy J. E. Phillips).

σ = reflection coefficient


LUMEN
(1000 mOsm)
diffusion H_2O
>1000 mOsm
Formation of Vesicles
Solute Transport
<400 mOsm
Secretion
diffusion H_2O
> 1000 mOsm
High Hydrostatic Pressure
laminar flow H_2O
Solute Transport
(400 mOsm)
HAEMOLYMPH
Barrier I
$\sigma = 1$
Barrier II
$\sigma \cong 0$
back diffusion of cation
H_2O by electroosmosis
transport of cation
+
−
Intracellular
(400 mOsm)
REVERSE PINOCYTOSIS
CO-DIFFUSION
ELECTRO-OSMOSIS

pump located on the blood side of the cell, an inward diffusion of water from the lumen could occur. If hypertonic cytoplasmic fluid became enclosed by a membrane and only the salts were pumped out of the vesicle, then if the vesicle coalesced with the serosal or hemocoel border, water could be moved with no net movement of salt. This hypothesis is similar to the one proposed by Pappas and Brandt (1958) for the production of hypotonic fluid by contractile vacuoles of protozoa.

The second hypothesis proposed in Fig. 1 also requires a hypertonic compartment maintained by a local ion pump. It is similar to a hypothetical situation discussed by Patlak (1963). Under this hypothesis of co-diffusion, water diffusing into the hypertonic compartment from the lumen would create a positive hydrostatic pressure within the compartment. If the blood side of this compartment had large pores with a low reflection coefficient (Staverman, 1948), while the lumen side had small pores, a situation would arise whereby the hydrostatic pressure gradient would push the solution through the large pores into the blood space. The ions would then be returned to the compartment by the local salt pump.

An objection to these first two theories is their requirement for a hypertonic cell. Phillips (1961) found the rectal tissue to contain low ionic concentrations of sodium, potassium, and chloride, totalling only 400 milliosmoles/l. Possibly hypertonicity is achieved by high

concentrations of ions or organic solutes other than those measured or the hypertonic compartment might not represent the whole of the rectal epithelium. This latter possibility is considered in the section dealing with electron microscopy.

The third theory does not invoke a hypertonic compartment. Since Phillips (1961) measured a net trans-rectal potential of 20 millivolts (lumen positive), cations being pumped into the lumen by the hypothetical local ion pump, would tend to diffuse back down the potential gradient to the blood side, moving water by electro-osmosis. The ions could be recycled by the ion pump located at the luminal border.

Each of these three hypotheses requires an active salt pump to drive the water movement. A test of these suggestions might be achieved by determining the degree of dependence of water movement upon salt transport, possibly by inhibiting salt transport with a specific inhibitor of the sodium-potassium pump, such as ouabain, or by the differential effects of other inhibitors (potassium cyanide, dinitrophenol, iodoacetate) upon the two processes of water and salt movement. If water transport occurred while ion transport was inhibited by ouabain, then its dependence upon sodium-potassium transport would be questioned. An object of this thesis was to study possible relationships between ion and water absorption.

There is a general lack of information on the ultra-structure of the insect rectum. Before any detailed discussion

of the water transport mechanism can be documented, it is necessary to examine the ultrastructure of the rectum to discover what systems of membrane barriers and compartments exist between the rectal lumen and the hemocoel across which large osmotic gradients may be maintained. A study of the general organization of the epithelium, by electron microscopy was therefore undertaken.

SECTION I. ULTRASTRUCTURE OF THE LOCUST RECTUM.

Introduction.

The importance of the insect rectum in maintaining water and salt balance has been known for some time (see Introduction). Only a limited amount of work, however, has been done to relate the ultrastructure of the rectum to its physiology. Clearly any realistic hypothesis concerning cellular mechanisms of rectal reabsorption must consider the system of membranes and compartments making up this organ. The studies of Noirot and Noirot-Timothée (1960) on Anoplotermes sanctus Silv., Smith and Littau (1960) on Macrosteles fascifrons Stål, and Bacetti (1962) on Aiolopus strepens Latr., indicates that rectal tissues possess many features common to other tissues known to be engaged in transport. Such tissues as the Malpighian tubules of insects, mouse kidney tubule, and crayfish nephridium, consist of columnar cells with infolded plasma membranes closely associated with numerous mitochondria (reviewed by Schmidt-Nielsen, 1963). However, the photomicrographs of the above workers who have studied other insect recta do not provide detail sufficient to support or refute any hypothesis regarding the mechanism of water transport in the locust, particularly since it is still not known how consistent rectal ultrastructure is throughout the insecta. The locust rectum produces a very hypertonic excreta. Any hypothesis must consider where the osmotic barrier is located in the tissue. From light micro-

scope studies, a secondary layer of cells was noted at the base of the columnar cells, but it was not known whether these cells formed a complete layer (Phillips, 1964), representing a second permeability barrier. Nothing was known of these cells' function or ultrastructure. It was hoped that information regarding continuity and function of these cells might be obtained.

A third object of the electron microscopic investigation was to test one of the hypotheses forwarded in the introduction. If reverse pinocytosis were involved in water transport, vesicles might be expected to occur at the serosal border of the columnar cells, and should be apparent under the electron microscope.

Materials and Methods.

Rectal tissue from fifth instar and adult *Schistocerca* was fixed for electron microscopy with Caulfields' buffered osmium (Kay, 1965), or with gluteraldehyde and post fixing in osmium (Sabatini, 1963). Dehydrated tissue was embedded in Maraglass and sectioned on a Porter-Blum ultra microtome. Thin sections were placed on acetone-cleaned, uncoated grids, and stained in uranyl acetate, (5 min.) and lead citrate (5 min.). The material was observed under an Hitachi HU-11-A electron microscope.

Observations.

(a) General organization.

The rectum consists of six longitudinal pads of

columnar tissue, as seen in the cutaway diagram in Fig. II and Plate 1. The pads are separated by low cuboidal epithelium (Phillips, 1961). The organ is supplied with numerous large tracheae, which branch profusely within the pads. The light micrograph in Plate 1 shows a cross section of the rectum. The columnar cells and secondary cells making up the rectal pad are indicated.

(b) The columnar epithelium.

Electron micrographs indicate that the columnar tissue consists of long (100 u) narrow (5-10 u) cells, as seen in Plate 2. The plasma membrane forming the luminal border is extensively folded, (Plate 3 and 4), greatly increasing the surface area. The folds, or cytoplasmic lamelli, are 800 to 1200 Å across and extend 3 or 4 u into the cell. These measurements agree with those of Smith and Littau (1960) on another insect, the leafhopper Macrosteles fascifrons.

The cytoplasm in the lamelli contains many mitochondria, (Plate 5 and 6), some of which extend almost to the luminal surface. The mitochondria appear in various conditions, some having cristae others containing a slightly granular matrix. The lack of cristae may be due to poor fixation, but with either gluteraldehyde or osmium, the appearance was the same. Mitochondria in the photographs of Bacetti (1962) had similar characteristics.

Microtubules are also seen within the cytoplasm

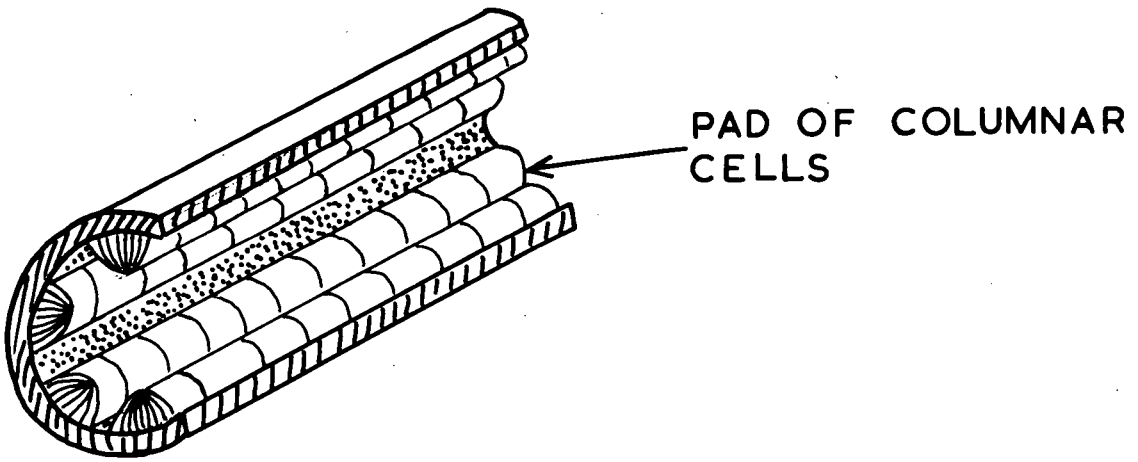
Fig. II.

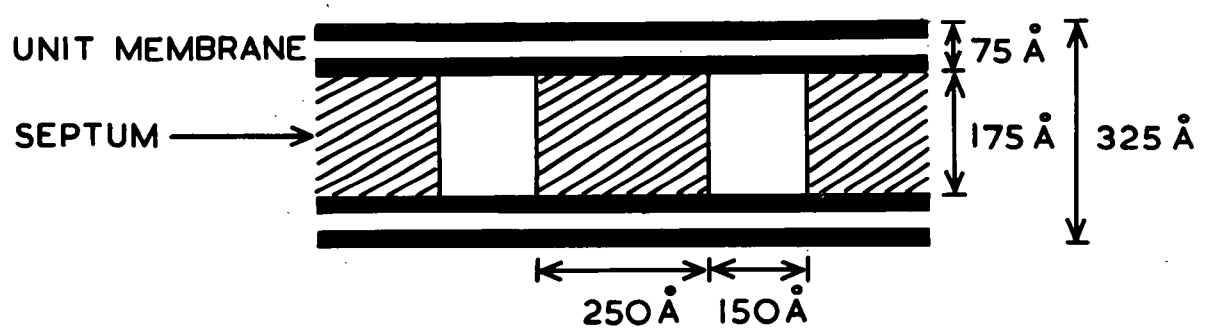
Cut away diagram showing lumen of locust rectum.

Fig. III.

Diagram showing septate desmosome.

(taken from Plate 8).


PAD OF COLUMNAR CELLS


UNIT MEMBRANE
SEPTUM
75 Å
175 Å
325 Å
250 Å
150 Å

of the lamelli, extending distally for an undetermined distance into the cytoplasm. Plate 3 shows the large number of tubules present, and their extension into the cytoplasm. The external diameter of 275-300 Å agrees with measurements described by Fawcett (1966). Although the true length was not determined, the tubules appeared at least 6-8 u long.

The intercellular border near the luminal border of the rectum shows cell contact phenomena similar to that which Fawcett (1966) terms a junctional complex. This consists of a zonula occludens and a macula adherens, followed by a region of desmosome. The desmosome in this case is a septate desmosome which is often found in invertebrates (Locke, 1965).

As shown in Plate 3, 8, and 9, the region of the septate desmosome takes a tortuous path, so that the borders of the two cells interdigitate like a dovetailed joint, locking the cells together. Mitochondria are often contained within the interdigitating extensions of cytoplasm, and are regularly seen clustered along the intercellular border. The septate desmosome, upon higher magnification (Plate 8.), shows an electron dense matrix between the two cell membranes. The characteristics of the septate desmosome taken from Plate 8 are shown in Fig. III. Periodically the septa are absent for a few hundred Angstroms, whereupon the two unit membranes fuse in what Fawcett (1966) calls a maculae occludents. (Plate 8.).

Beyond the region of the desmosome, towards the

hemocoel, the intercellular border widens to 400 Å and often separates completely leaving intercellular gaps. (Plate 3 and 10). These gaps are very prominent towards the haemocoel side of the cell. The border from the desmosome to the blood side is very tortuous and has large numbers of mitochondria closely applied to it. Although more evidence is needed, it is strange that in the twelve micrographs examined the intercellular border has not been observed to join the serosal or hemocoel border of the cell. It often stops just short of the border, and may even terminate in a vesicle or intercellular gap. (Plate 11).

At the serosal border, numerous oval vesicles are noted, usually .5 to 1.0 u long. These have not been observed to rupture, and always appear with a fringe of cytoplasm between the vesicle membrane and the plasma membrane. (Plate 11).

Other cellular inclusions are lysosomes, similar to those described by De Duve (1966) and Fawcett (1966), and tracheoles. Lysosomes are present in nearly every plate, (labelled in Plate 10). The tracheoles are seen in Plate 3, but are found in many regions of the cell, accompanied by their surrounding cell, containing mitochondria and occasionally a nucleus. It may be to these small nuclei that Phillips (1961) and others refer to as appearing in the proximal cytoplasm of the columnar cell, near the lumen.

(c) Basement Membrane.

Between the columnar epithelium and the secondary cells is a 1.6 u thick basement membrane consisting of many 200 Å fibers in a complex array. The length of the fibers, though undetermined, is greater than 80 u. Periodic striations were not discernable.

(d) Secondary Cells.

Between columnar cells and the muscle are the secondary cells. These ovoid cells (20 u x 10 u) have a peculiar border of spongy appearance, containing many cavities from which 300 Å channels lead into the cell. (Plate 12, 13, and 14). The channels twist back and forth around many mitochondria, and extend into the cell to the level of the nucleus. These channels and mitochondria completely fill the cytoplasm of the secondary cell on the side proximal to the columnar cells. Distally the cytoplasm of the secondary cell contains some endoplasmic reticulum and a few scattered mitochondria, but no elaborations of the plasma membrane.

(e) The Sheath.

Between the secondary cells and the hemocoel is a basement membrane, the tracheal trunks, and a thin layer of muscle. (Plate 15.)

Discussion

The greatly increased surface area of the cytoplasmic lamelli, in conjunction with the large number of mitochondria located at the luminal border, indicates that this surface is responsible for some function requiring energy and a large working surface, a function such as transport. The observation that the luminal border has tight intercellular junctions is a further indication that this surface is a permeability barrier. Clearly, if a membrane were creating and maintaining an electrochemical gradient, it would be advantageous to reduce the passive permeability (back diffusion) via intercellular channels. It has been suggested that these junctional complexes (Farquhar and Palade, 1965) bind a series of individual cells into a structurally continuous barrier, across which an electrochemical gradient can more easily be maintained. The zonula occludents, for example, could restrict water movement along the intercellular spaces. Farquhar and Palade also suggest that the maculae occludents may represent a region of low resistance, allowing rapid equilibration of sodium and potassium ions between the cells, making the cell mass a functionally continuous compartment. Lowenstein et al. (1964) suggest that the septate desmosome may also be a low resistance pathway between cells from observations of electrical resistance and passage of fluorescein dye between adjacent cells. Of possible interest concerning the septate desmosome is the observation that the

septa appear to be separate inclusions between the unit cell membranes, in agreement with the Coggeshall (1966) findings in the earthworm epidermis, rather than continuations of the plasma membrane as suggested by Locke (1965). Whatever other functions the desmosome has, it probably binds the cells into a tight sheet, which is of primary importance if the cells are responsible for osmotic work.

The occurrence of mitochondria in such close association with the wanderings of the intercellular border is of unknown significance. (At low magnification, one sees that almost all the mitochondria in the cell are close to either the infolded luminal border or to the two intercellular borders of the cell. See Plate 2.)

The function of the microtubules in this tissue is unknown. Fawcett (1966) suggests that microtubules have a role in maintaining cell shape as a "cytoskeletal element". If true, this would be of advantage in shaping and maintaining the cytoplasmic lamelli, thus maintaining the increased surface area for transport. An alternative, though not mutually exclusive suggestion is that the tubules may be important in protoplasmic movements, as implied by Porter (1966). His observations suggest that transport does not take place within the tubules but he did observe, by cinephotography, cytoplasm and mitochondria streaming along the surface of the tubules. In a study of spindle fibers and microtubules, Mazia (1966) analyzed the tubules

biochemically and suggested that they might be composed of actin. He also states that microtubules could be found nearly everywhere that movement occurs within the cell. If the function of the tubules is one of cytoplasmic movement within the cytoplasmic lamelli of these rectal columnar cells, then it is conceivable that the diffusion rate of recently transported ions and water away from the membrane and deeper into the cytoplasm, may be augmented by the presence of microtubules. This would reduce the possibility of back diffusion of ions and would result in an increased efficiency of the transporting system.

The lysosomes, by their suggested function as autophagic vesicles (De Duve, 1966), could be significant to these cells which have a high rate of metabolism, and make heavy demands on the mitochondria. Presumably the autophagic vesicles remove and digest old mitochondria, making their components available for reincorporation into new mitochondria. Mitochondrial fragments are seen in lysosomes in some of the micrographs.

Several general similarities exist between rectal tissue of the locust and other tissues known to be engaged in transport. Osvaldo and Harrison (1966) have shown microtubules between the infoldings in cells of the loop of Henle in mamalian kidney. Fawcett (1966) shows lysosomes in kidney tubule cells. A tight junctional complex is seen in Malpighian tubules (Tsubo and Brandt, 1963), amphibian skin (Farquhar and Palade, 1965), and hamster

intestinal epithelium (Fawcett, 1966). All are tissues across which transport takes place and which require a good tight barrier to reduce permeability. Finally, the infolded border associated with numerous mitochondria is, of course, common to transporting surfaces.

One apparent difference between rectal columnar epithelial cells and those of the kidney tubule, Malpighian tubule of insects, crayfish nephridia, and other excretory tubules, is the lack of the infolding of the basal membrane. In many cases, this is the area of heaviest infolding of the plasma membrane, just the opposite of what has been found in the locust. Of historical note, Pease (1956) observed that tissues engaged in water transport had the distinctive characteristic of the infolded serosal border. Locust rectum, unusual in its ability to transport water, does not have these infoldings. It is now known that the tissues Pease chose for his comparison were tissues where water movement accompanies ion movements passively and does not involve water transport against an increasing osmotic gradient.

The interesting observation that the secondary cells have a complex ultrastructure and exhibit a polarity of infoldings and mitochondria may be significant in the ability of the rectum to transport water. That the secondary cells may form a permeability barrier is indicated but not proven by the micrographs. If junctional complexes such as the desmosome had been observed, one might have been able to suggest an osmotically tight barrier across these

secondary cells. However, this was not observed. The highly infolded surface and the high concentration of mitochondria indicate a transport function for these cells.

Referring to the introductory model labelled co-diffusion, it is possible that the hypertonic compartment could be the space between the columnar cells and the secondary cells. The secondary cells could be the surface secreting salt into the compartment to maintain the hypertonicity, while the thin region between adjacent secondary cells might represent the area of large pores with a low reflexion coefficient. Hypothetically, this could pump water as proposed in the introduction.

An alternative hypothetical function of the secondary cells might be as a second stage ion pump in series with the large columnar cells. Of possible interest is the finding of Jarial (personal communication) that the corixid, Cenocorixa bifida (Hung.), which excretes hypotonic urine only, has no secondary cells.

In summary, this study of the ultrastructure of the locust rectum does not rule out the hypothesis of water transport by reverse pinocytosis, given in the introduction, since vesicles are observed on the haemocoel side of the columnar cells.

The discovery of a complex secondary cell layer with its polarity of infoldings and distribution of mitochondria, indicates that two cell layers in series are involved in transport, but whether one or both are involved in transporting water remains to be discovered.

KEY TO ABBREVIATIONS USED IN PLATES 1 - 15.

In order of appearance:

L	cytoplasmic lamellae
D	desmosome
T	microtubule
IG	intercellular gap
Tr	tracheole
M	mitochondrion
Ly	lysosome
V	vesicle
B	basement membrane
C&M	channels and mitochondria
N	nucleus
Ep	columnar epithelium
Sec	secondary cell
Mus	muscle

PLATE 1.

Light micrograph, showing a cross section of the rectum of the desert locust. Fixed in Bouin's, and embedded in wax.

(x80)

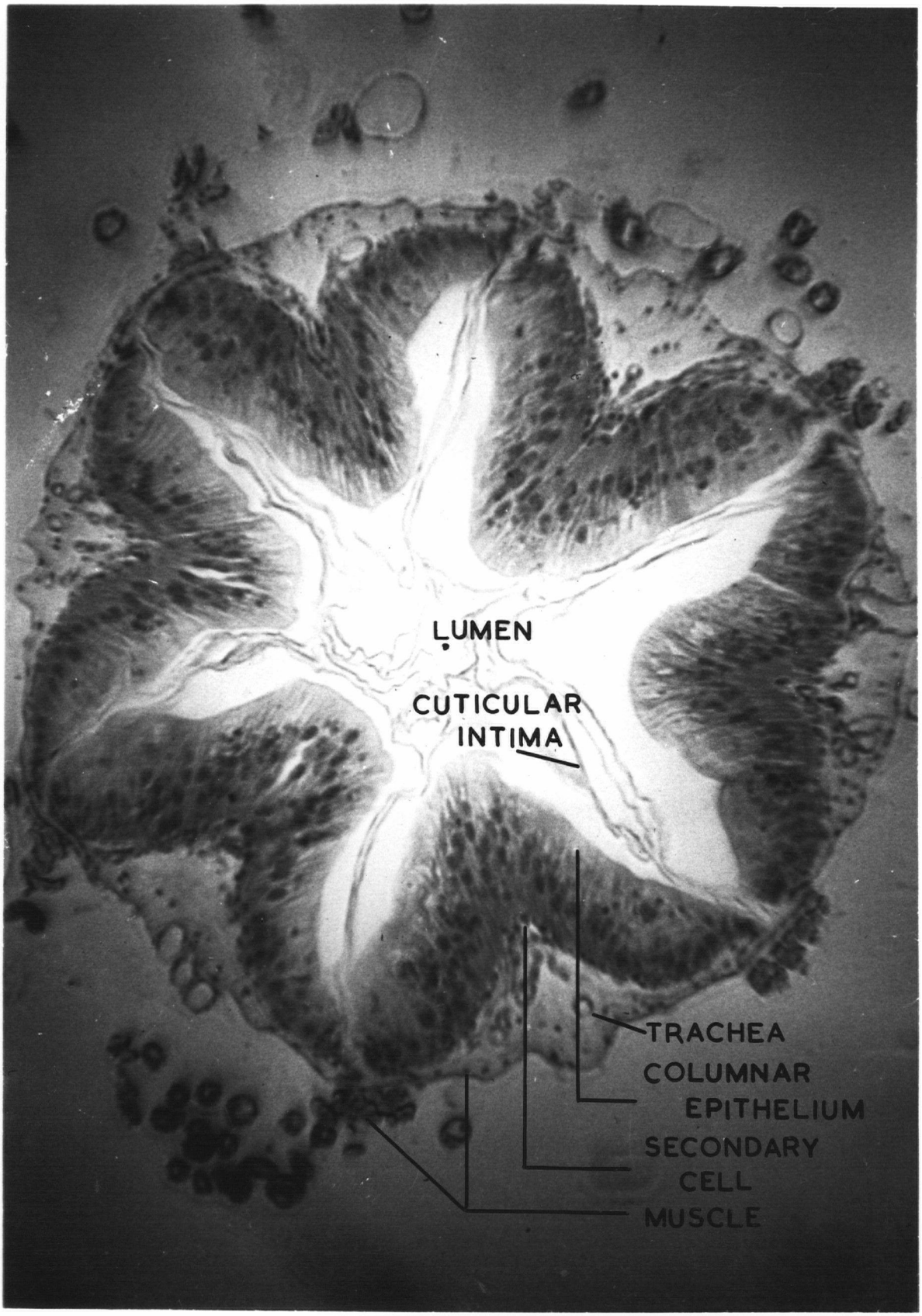

LUMEN
CUTICULAR
INTIMA
TRACHEA
COLUMNAR
EPITHELIUM
SECONDARY
CELL
MUSCLE

PLATE 2.

Diagram of rectal epithelial and secondary cells in region of rectal pads, composed from electron micrographs.

(x150)

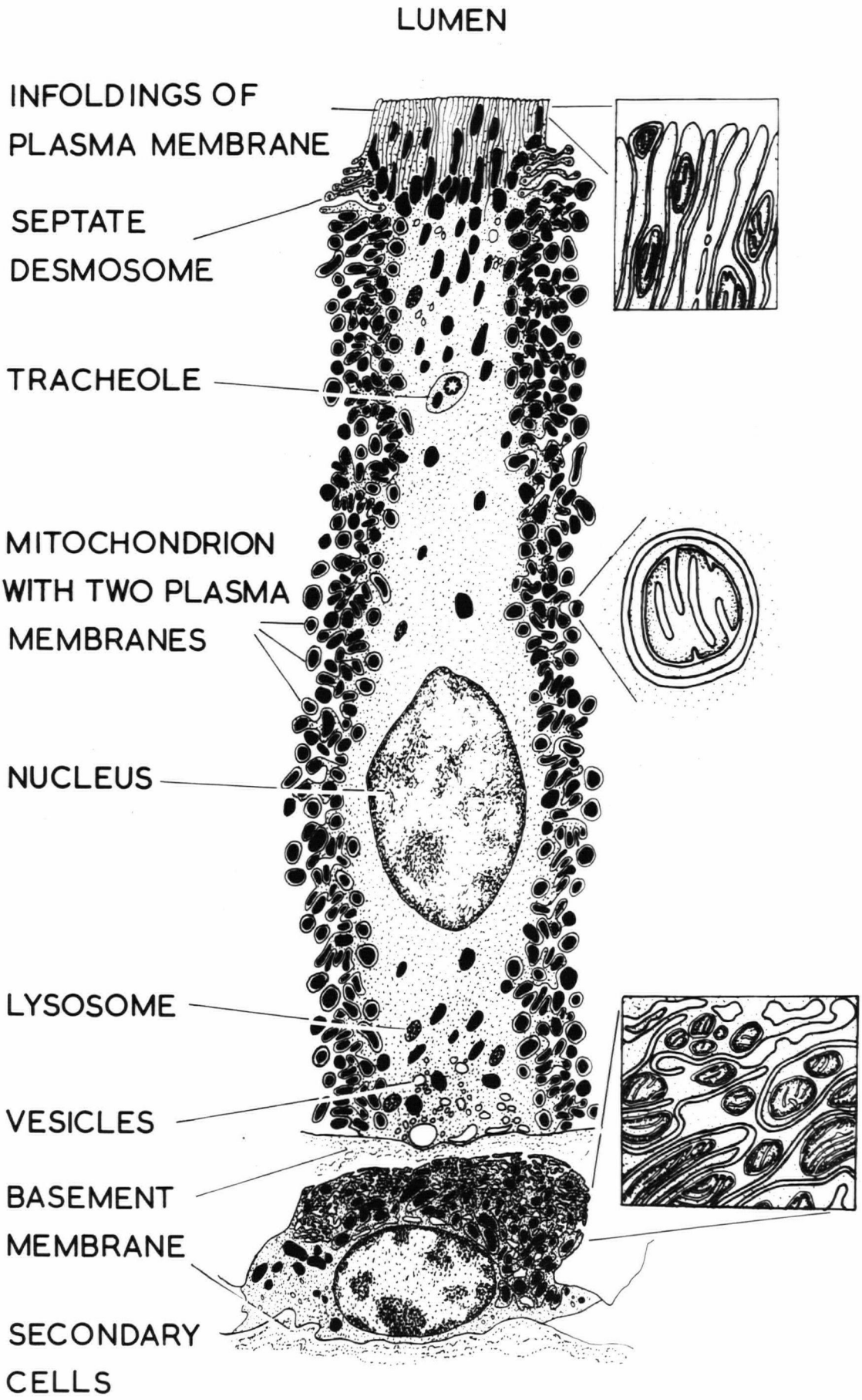

LUMEN
INFOLDINGS OF PLASMA MEMBRANE
SEPTATE DESMOSOME
TRACHEOLE
MITOCHONDRION WITH TWO PLASMA MEMBRANES
NUCLEUS
LYSOSOME
VESICLES
BASEMENT MEMBRANE
SECONDARY CELLS

PLATE 3.

Micrograph showing the luminal border of the rectal epithelium. L---cytoplasmic lamelli; D---desmosome; T---microtubules; IG---intercellular gap; Tr---tracheole.

(X 7,200)

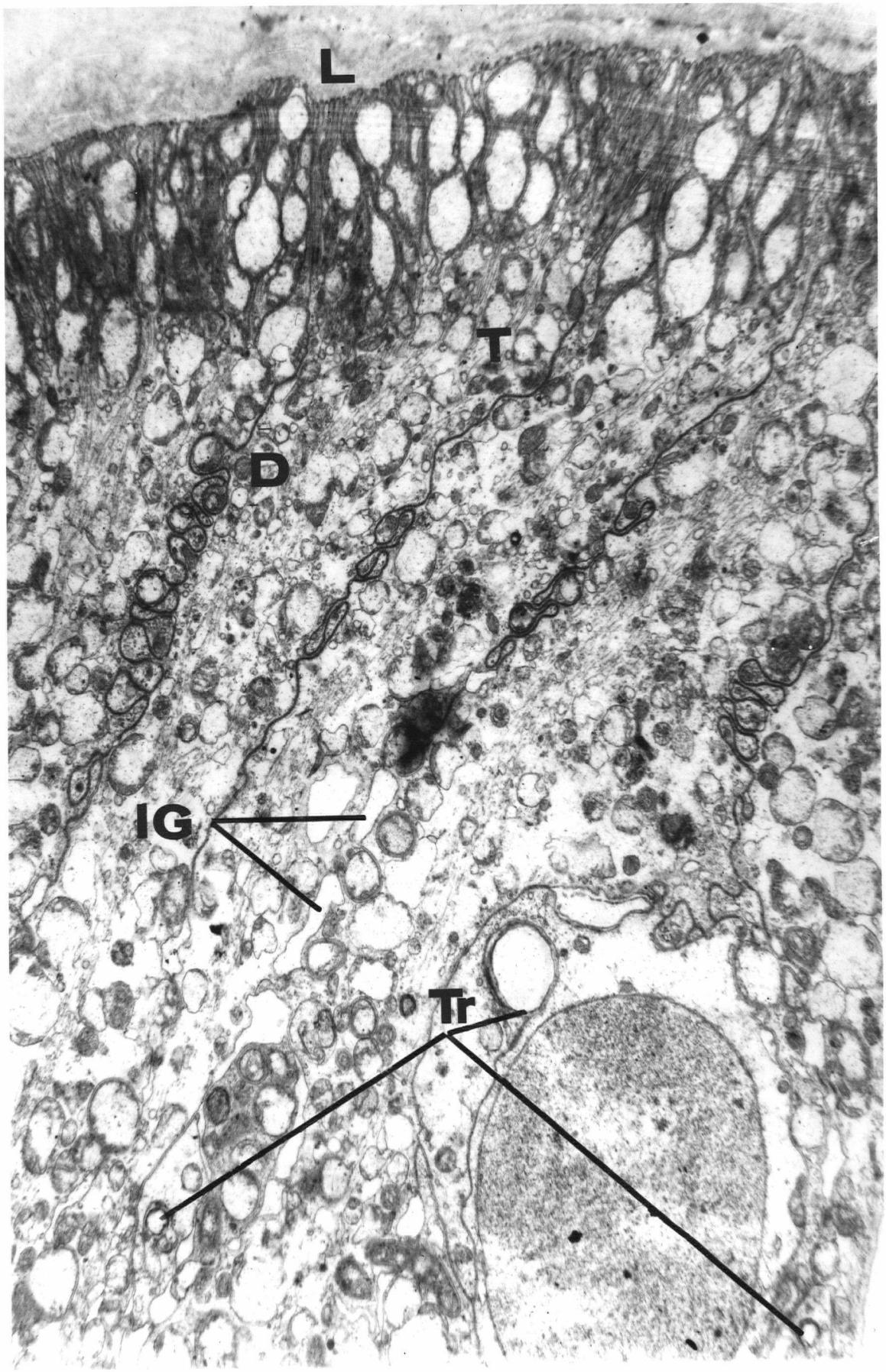
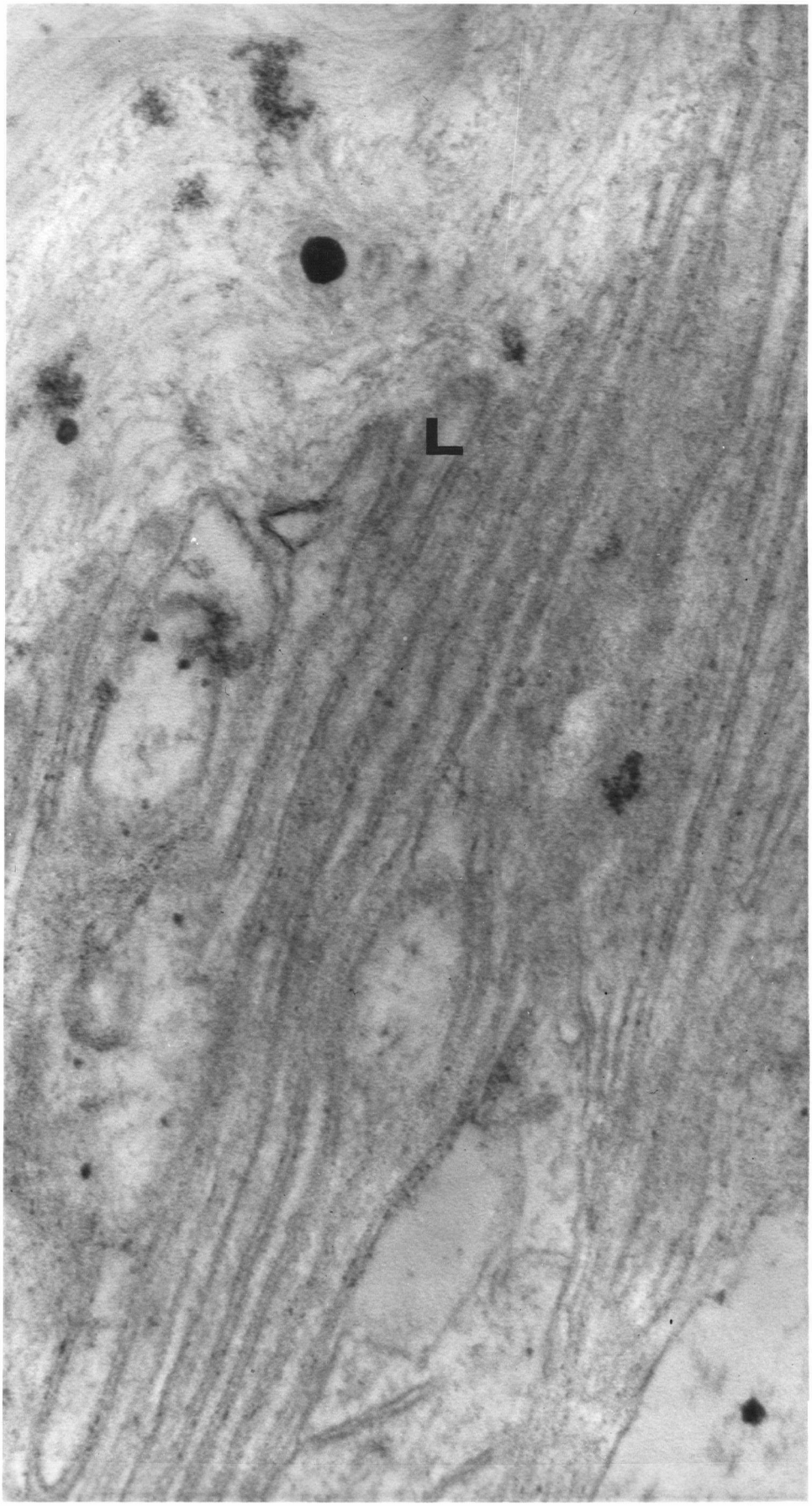

L
T
D
IG
Tr

PLATE 4.

Micrograph of cytoplasmic lamelli. Note density of these infoldings. L---cytoplasmic lamelli.

(X 75,000)



4

PLATE 5.

Micrograph showing the occurrence of mitochondria (M) within the cytoplasmic lamelli.

(X 22,000)

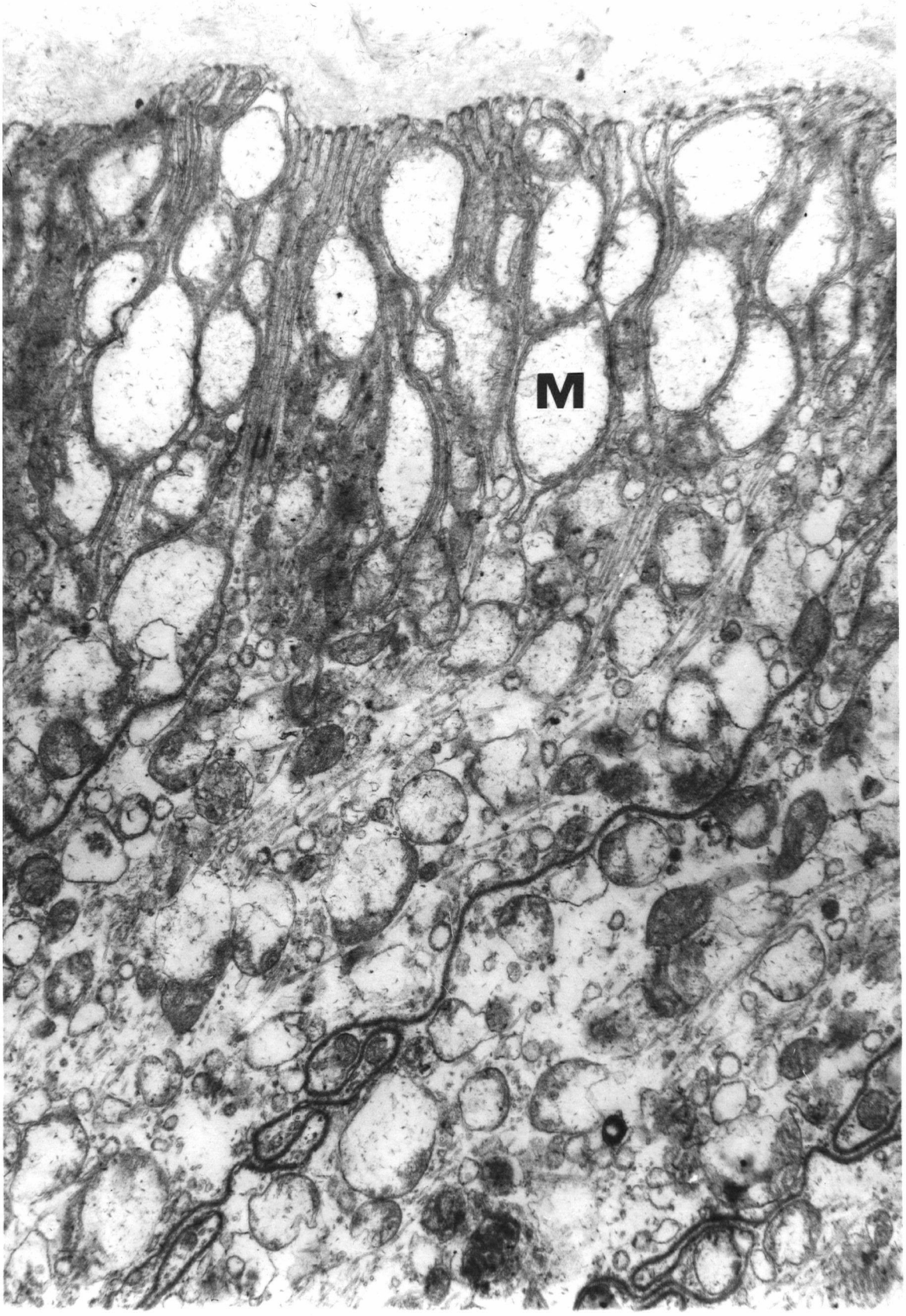

M

PLATE 6.

Micrograph showing how some mitochondria appear to have normal cristae. The difference between microtubules and the cytoplasmic infoldings can clearly be seen.

(X 60,000)

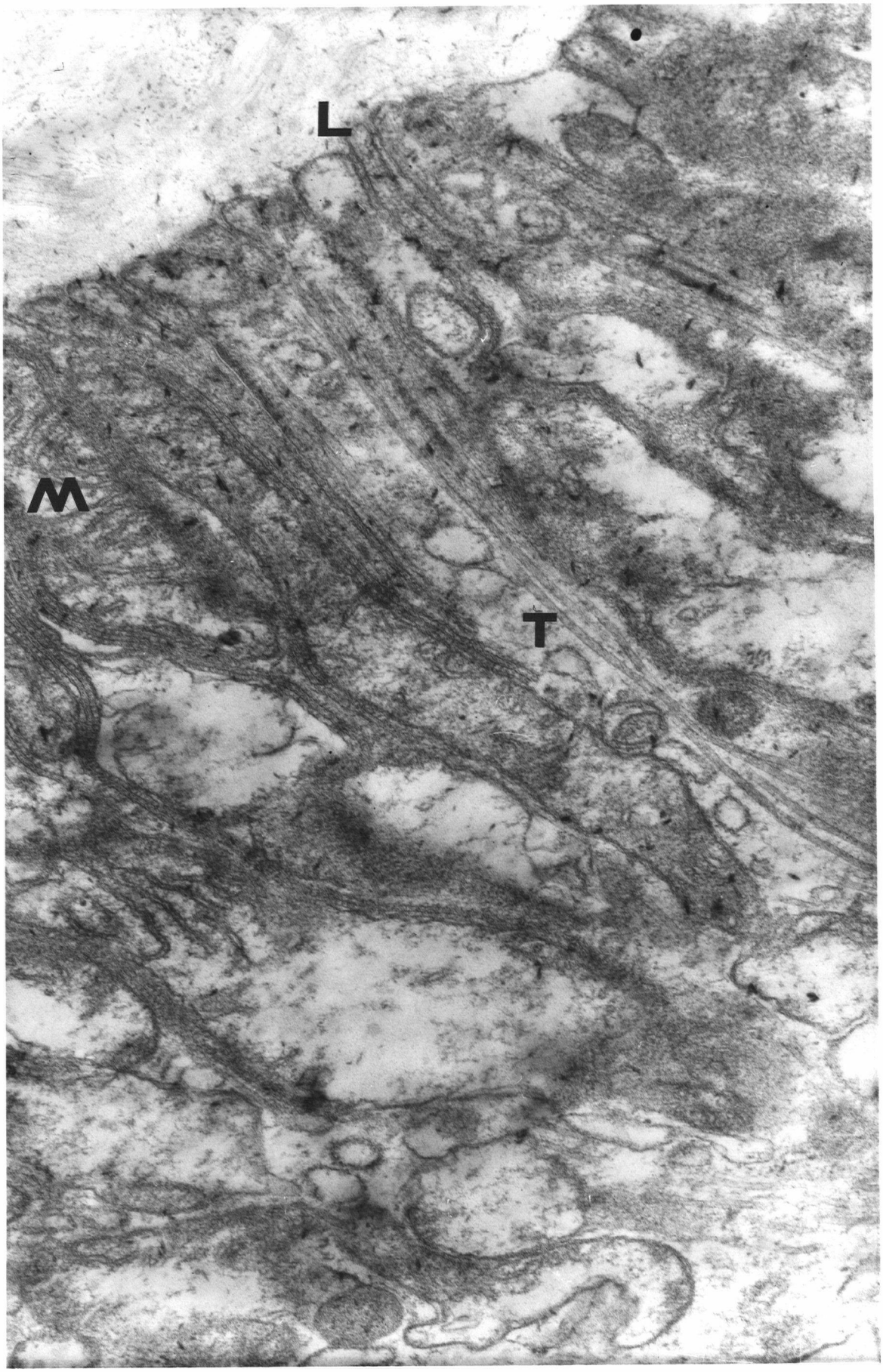

L
M
T

PLATE 7.

Micrograph showing number of microtubules within the cytoplasmic lamelli. Note the striated appearance at left.

(X 40,000)

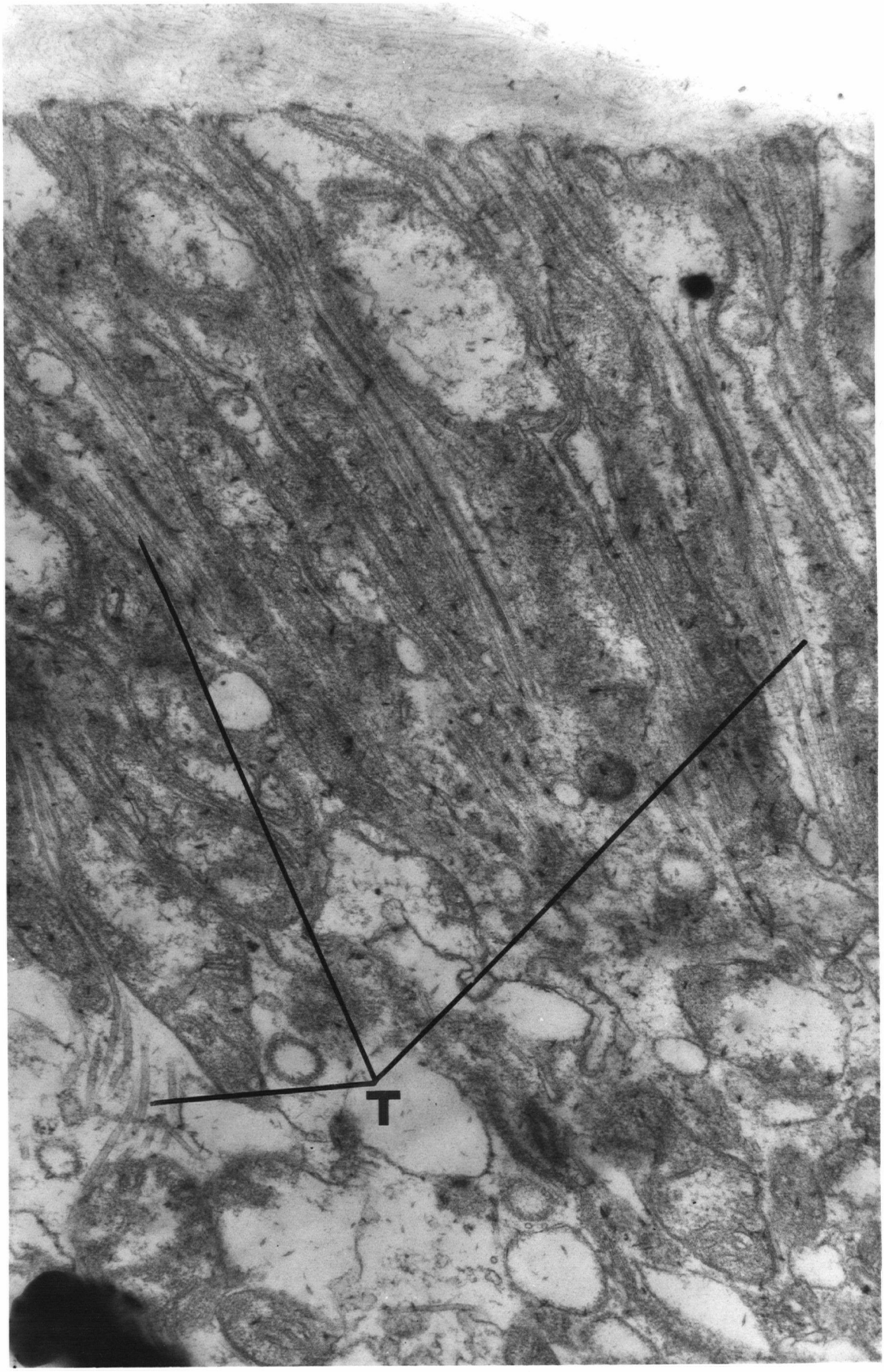

T

PLATE 8.

Micrograph showing septate desmosome. Septa may be seen at D and at arrow. A macula occludens may be seen below and to the right of D, where the two unit membranes fuse into a 5 layered system.

(X 50,000)

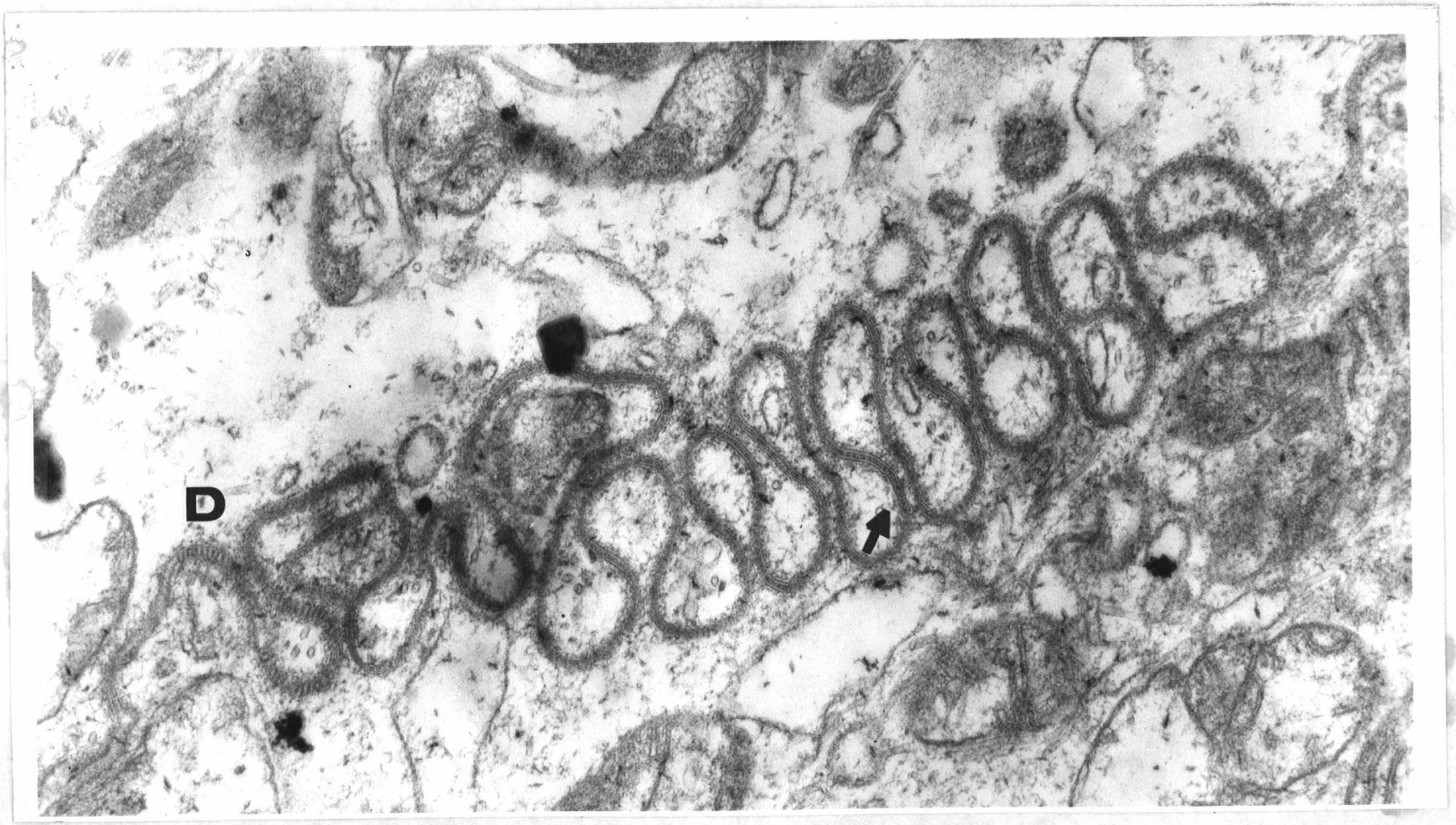

D

PLATE 9.

Micrograph showing how many of the mitochondria appear within the interdigitations of the intercellular border.

(X 75,000)

PLATE 10.

Micrograph showing the serosal or hemocoel border of the columnar cells. Ly---lysosome; IG---intercellular gap; V---vesicle; M---mitochondrion; B---basement membrane; Tr---tracheole.

(X 10,000)

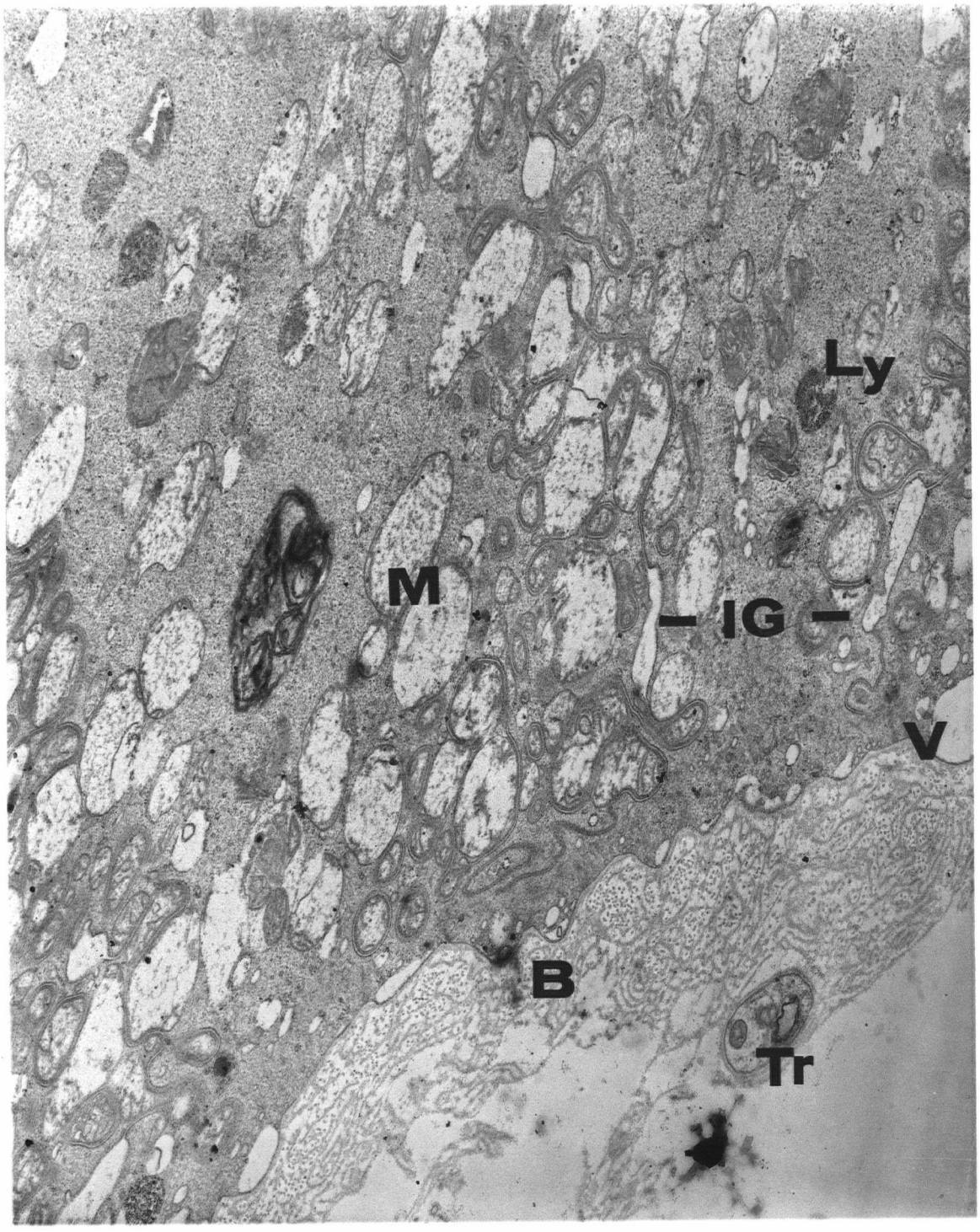

Ly
M
IG
V
B
Tr

PLATE 11.

Micrograph showing the intercellular border approaching the distal border of the cell. The vesicle is at the cell border but it has not been established that these are secreted.

(X 75,000)

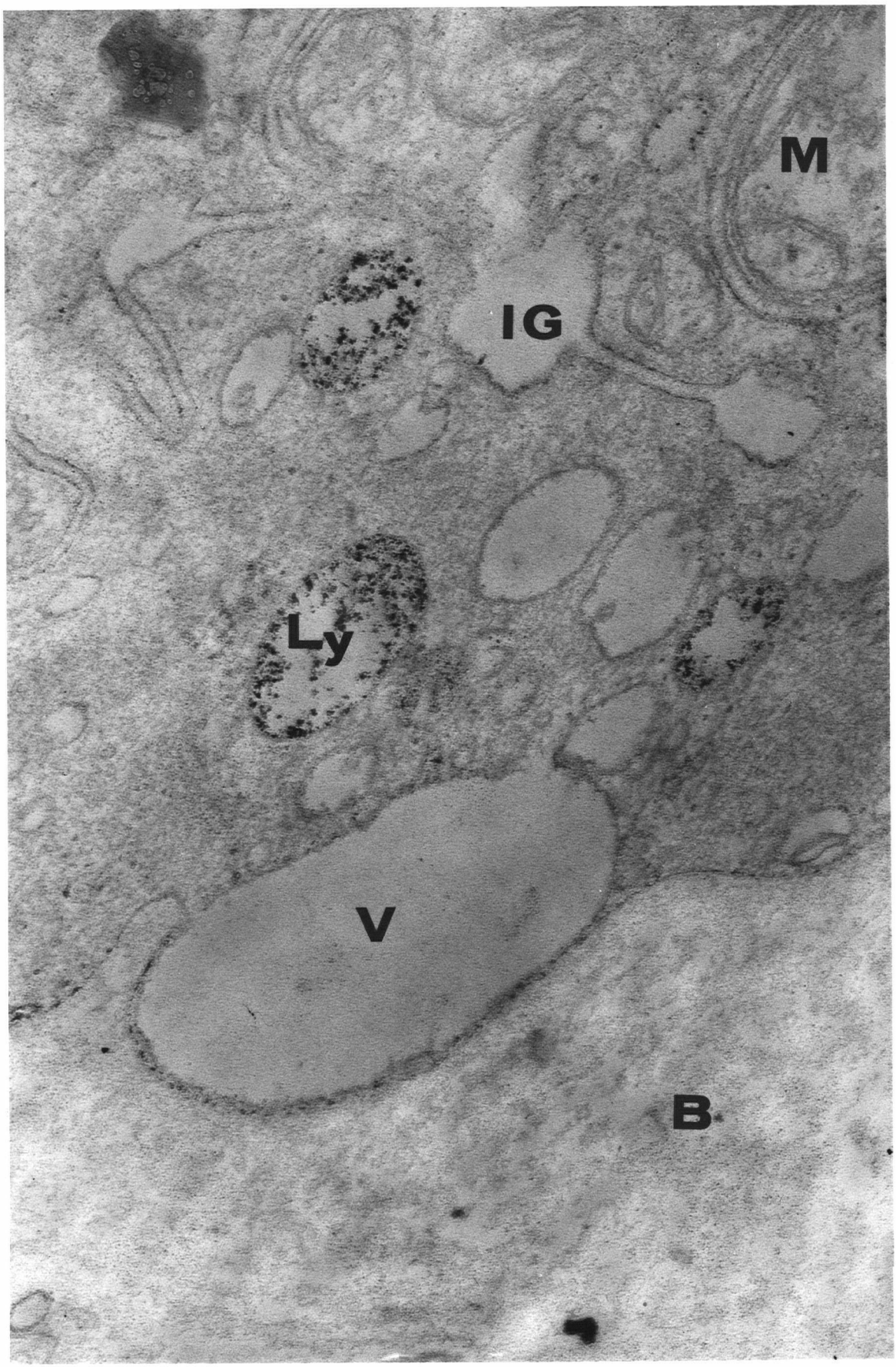

M
IG
Ly
V
B

PLATE 12.

Low power micrograph of a secondary cell showing the channels and mitochondria (C & M) in the spongy like region (top) of the cell, on the lumen side. The nucleus (N) and a tracheole are also seen. (Tr.)

(X 4,500)

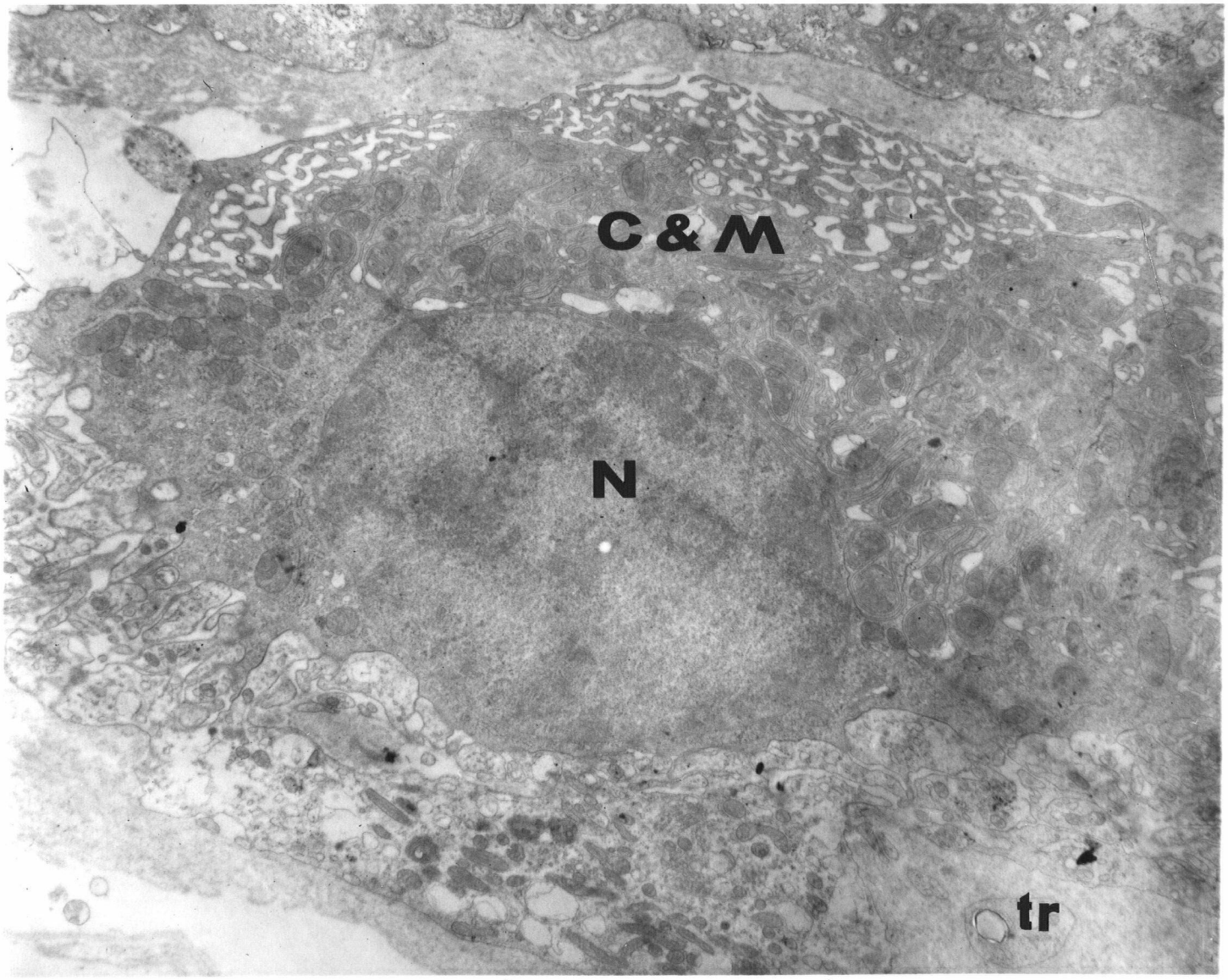

C&M
N
tr

PLATE 13.

Micrograph showing the sponge-like appearance of the border of the secondary cells.

Ep---columnar epithelium; B---basement membrane; Sec---secondary cell.

(X 18,000)

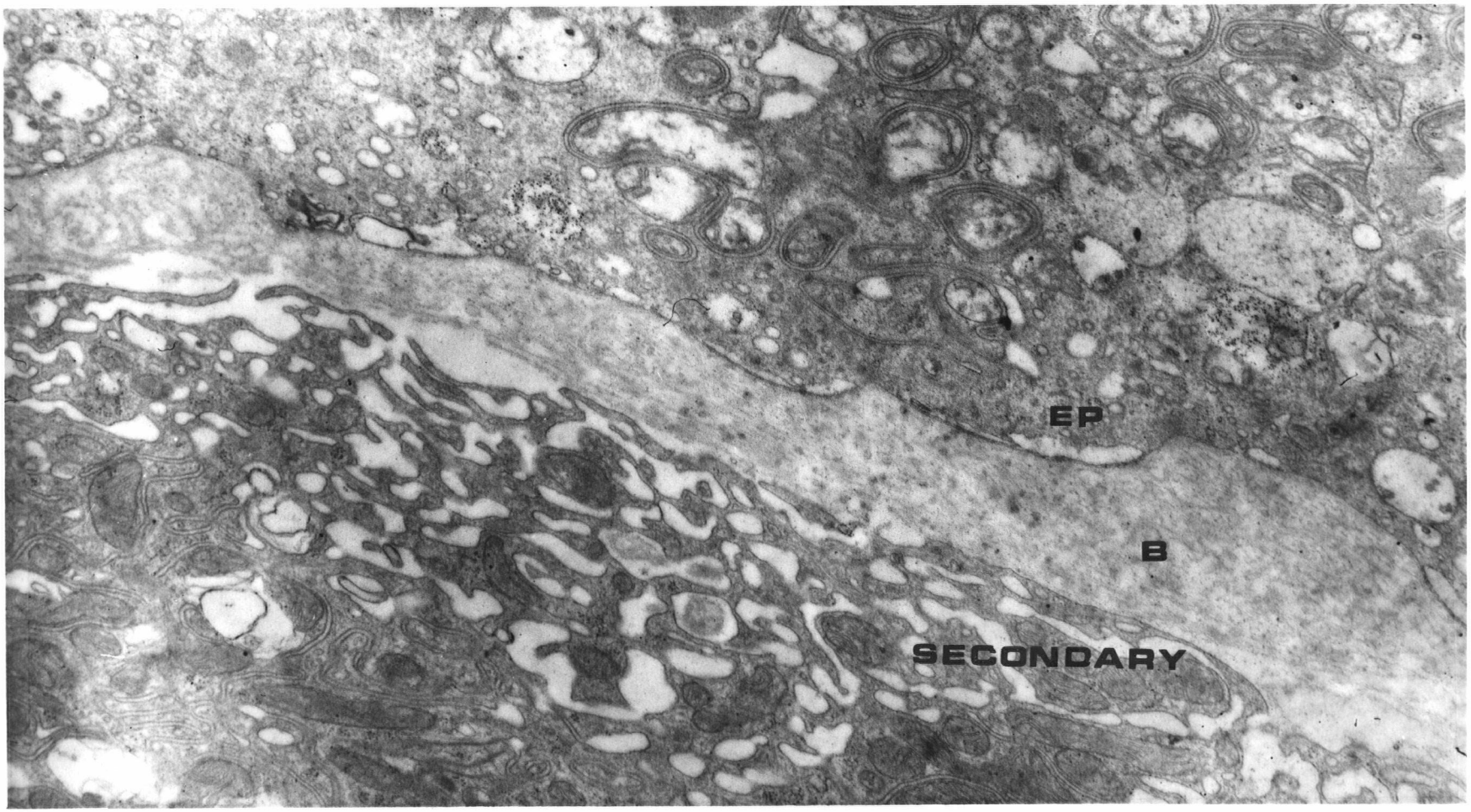

EP
B
SECONDARY

PLATE 14.

Micrograph showing some detail of the channels (C) and mitochondria (M) in the spongy border of the secondary cell.

(X 76,000)

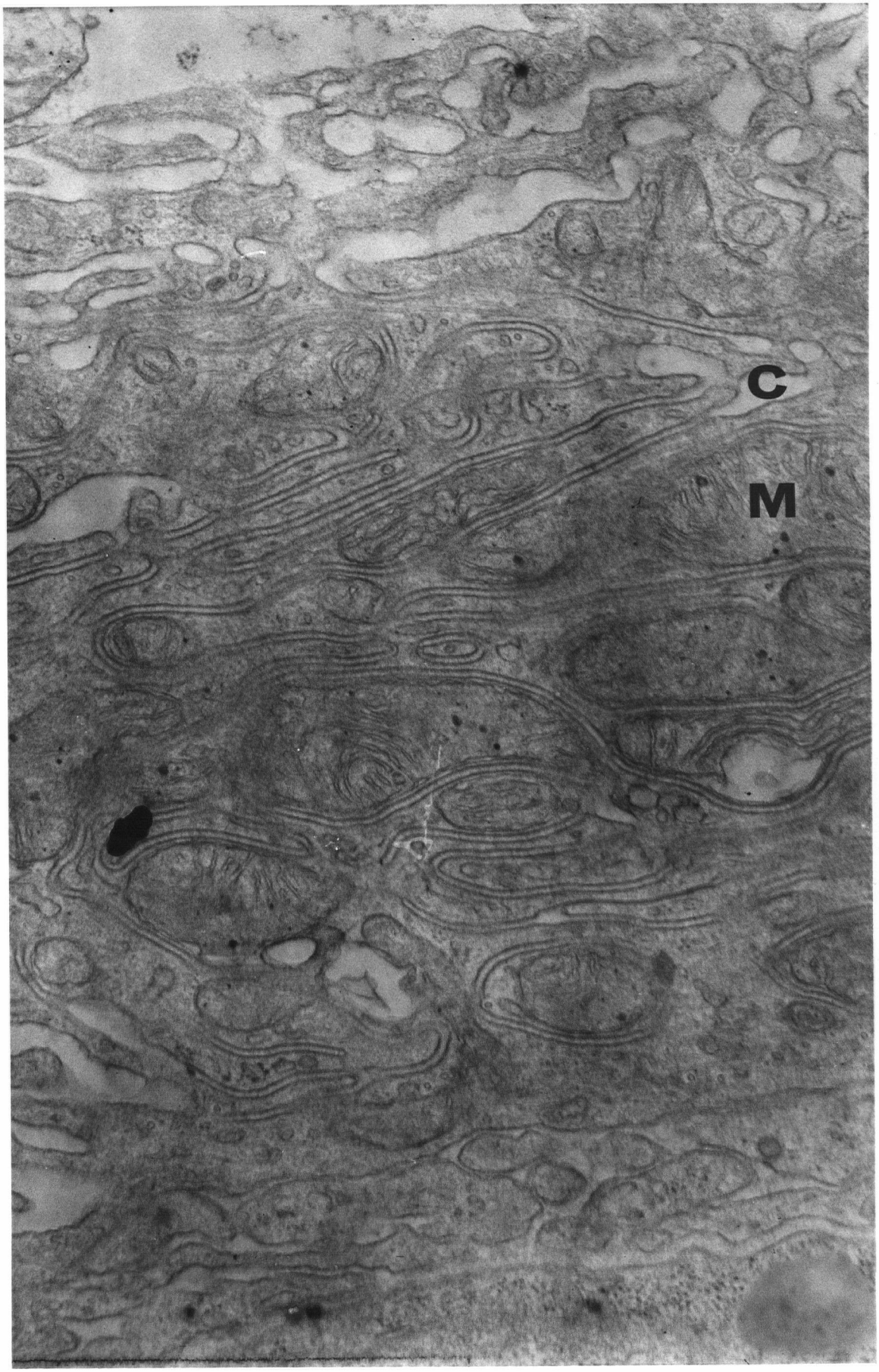
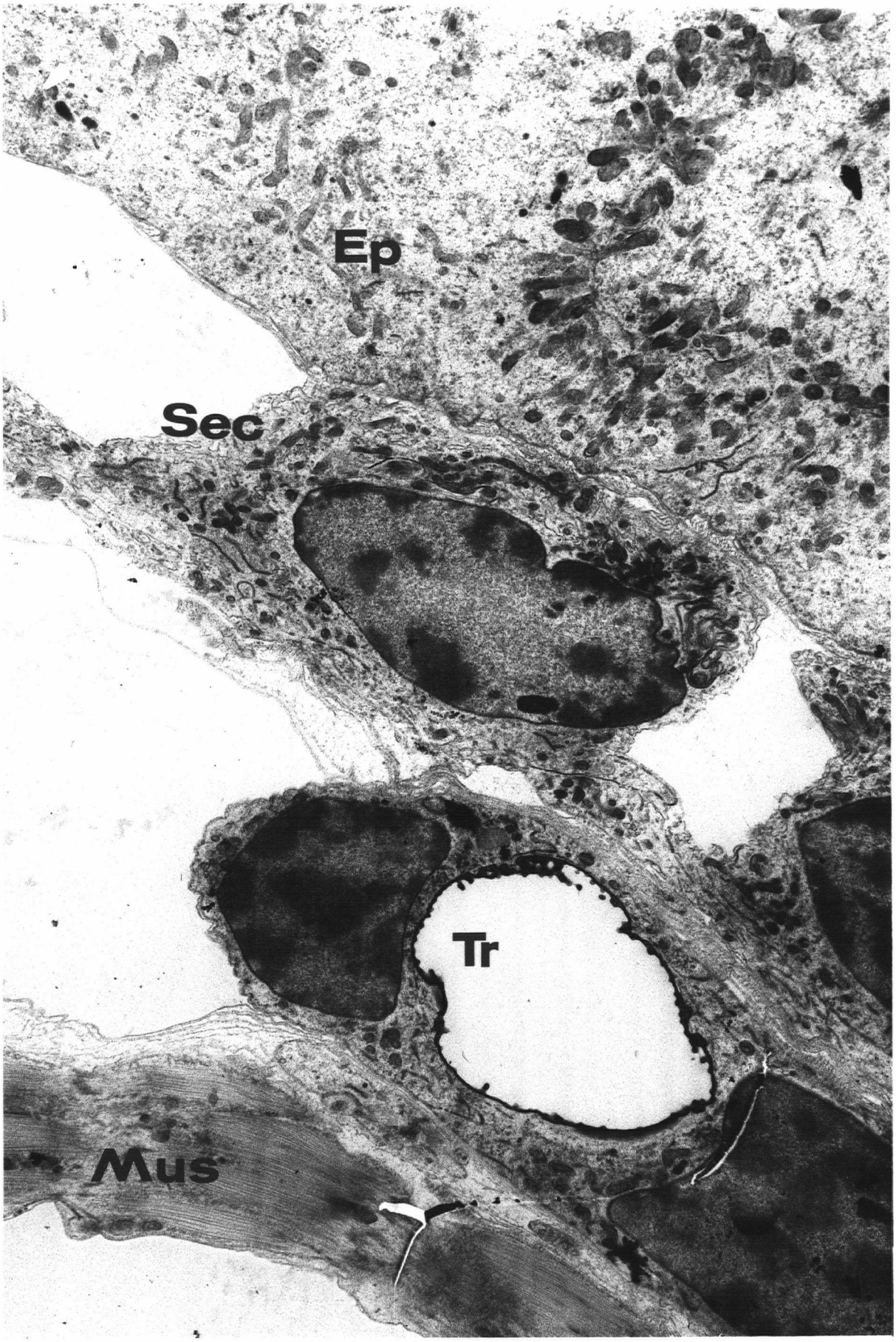

C
M

PLATE 15.

Micrograph giving the relationship between the epithelium (Ep), the secondary cell (Sec), and the trachea (Tr) and muscle (Mus). Note the narrow junction of the two adjacent secondary cells. This was from a section of the fifth instar rectum, so that the inclusions are somewhat smaller than they appear in the adult.

(X 4,000)


Ep
Sec
Tr
Mus

SECTION II.

STUDIES ON IN VITRO PREPARATION OF RECTUM OF LOCUST

Introduction

The object of the present study was to develop an *in vitro* preparation of *Schistocerca* rectum, and to follow the movements of solute and water across the rectal epithelium. The dependence of these movements upon cellular metabolism, not previously shown, might then be assessed by use of inhibitors such as potassium cyanide, iodoacetate, and dinitro-phenol. The advantage of an *in vitro* preparation is that it permits a study of the direct effects of the inhibitors on metabolism, and on ion and water movements across the rectal epithelium, independent of secondary effects due to inhibition of respiratory ventilation and circulation present in the *in vivo* preparation.

The present investigation presented an opportunity for comparing the *in vitro* preparation with the *in vivo* studies of Phillips (1964). The comparison of *in vitro* with *in vivo* studies, not often made in the literature, allowed an evaluation of the performance of the *in vitro* preparation of the rectum. These experiments were also designed to confirm water transport using a different volume indicator (inulin-C^{14}) from that used by Phillips (albumin-I^{131}).

Development of a functional *in vitro* preparation makes possible experiments such as those of Ussing

(1954) on short-circuited frog skin, and simplifies the study of ionic fluxes. Such studies would greatly facilitate the elucidation of the cellular mechanisms involved in the water transport by locust rectum.

Materials and Methods

(a) Materials.

Mature, male Schistocerca gregaria were chosen from the laboratory colony, reared on a diet of bran, fresh lettuce, grass, and water in a controlled environment of 83°F. and 50% relative humidity under continuous light. To reduce the variation due to age, temperature, environmental history, and other variables, it was decided to run four tests (no inhibitor plus three experimentals with inhibitor) simultaneously on individuals from the same egg batch reared in a single cage. The control and test animals were roughly chosen for uniform size and weight.

(b) External and internal solutions.

For these in vitro experiments, it was desirable that the external medium (the solution bathing the outside of the rectal sac) be as similar to locust blood as possible. Schistocerca hemolymph contains some eleven amino acids, trehalose, dextrose, and the main blood ions (Treherne, 1958). A medium similar to that described by Treherne was prepared. This had an osmotic pressure comparable to that of the blood (380 milliosmolar). The pH of 5.4 was balanced to

7.0 (the pH of the hemolymph). On balancing the pH, a precipitate formed leaving the medium a milky colour. This was not filtered off.

The inhibitors were added to this external solution in concentrations of 10^{-2}, or 10^{-3} Molar. Inhibitors were not added to the internal solution (the solution injected into the lumen of the rectum) since the lumen of the rectum is lined with a cuticle permeable only to small molecules (Phillips, 1965). Early solutions contained penicillin and streptomycin, but this was later discarded with the finding that freezing the stock solution adequately inhibited bacterial growth. Subsequently, all solutions were stored at $-10^{\circ}C$.

Since the experiments were designed to test active transport out of the rectum, the composition of the internal solution was adjusted to set up electrochemical gradients favouring passive net flux of ions and water into the rectum; hence any absorption out of the rectum would be active. The internal solution consisted of 70 mM/l. sodium chloride, made hypertonic to the external solution by addition of 260 mM/l. sucrose. The final solution had a freezing point of $-0.75^{\circ}C$. and a pH of 6.5.

The volume indicator (inulin-C^{14}, New England Nuclear Corp.) was added to give the solution an activity of about 1000 cpm/ul.

As a visual aid in detecting leakage of the rectum, the dye amaranth was added (3×10^{-4}M.). In the

few cases of rectal puncture, this dye was immediately obvious in the external media.

(c) In vitro preparation of the rectum.

Schistocerca were sacrificed by removing the head. The abdomen was cut off near its base and placed in a simple holding device (Fig. IV), consisting of a short length of 1/8 inch dia. surgical tubing and pins, which spreads the abdomen, thus holding it in place on the wax operating table.

Cannulation of the rectum through the anus was accomplished by using a 20 mm length of a 30 ul pipette (Drummond Microcap) with the end annealed and lubricated with distilled water. This cannula was very easily inserted 3-4 mm into the anus, while holding the epiproct with forceps, and quickly fixed in place with a beeswax and resin mixture (Fig. IV).

An oxygen supply was fixed into the hemocoel by inserting a fine bore (#10) polyethylene tube through a small cut in the cuticle of the eighth segment. This was fixed in place with beeswax and resin (Fig. IV).

The rectum was then quickly dissected from the abdomen by making a lateral cut from the eighth segment, anterior through two segments (Fig. IV). A flap of cuticle was removed from the abdomen and the gut grasped with forceps and pulled out through the opening. The rectum was then cut just anterior to the rectal pads and the hindgut discarded. The rectal contents were thoroughly

washed out by coupling an eye-dropper to the cannula and flushing saline through the organ, followed by several aliquots of air. The cut end of the rectum was then ligated with cotton thread and the organ dissected out as cleanly as possible.

The sac was incubated in the external saline, with or without inhibitor, for one hour after which time the cannula was connected to the injection apparatus (Fig. V) and 30 or 50 ul of fluid injected into the ligated rectum. A 10 ul initial sample was taken for analysis after complete mixing of the injection solution with any residual rectal fluid. The sample could be easily removed by withdrawing the rod from the syringe (Fig. V) and inserting a fine pipette down into the fluid.

The injected fluid was left in the rectum for 5 hours following incubation, except where noted. The final sample was taken by disconnecting the coupling and cannula, and gently squeezing the remaining rectal fluid from the rectum onto a paraffin surface. The squeezing was necessary in the cases where volume reduction took place, since only about 2/3 (14 ul) of the initial content remained.

(d) Storage of rectal fluid sample.

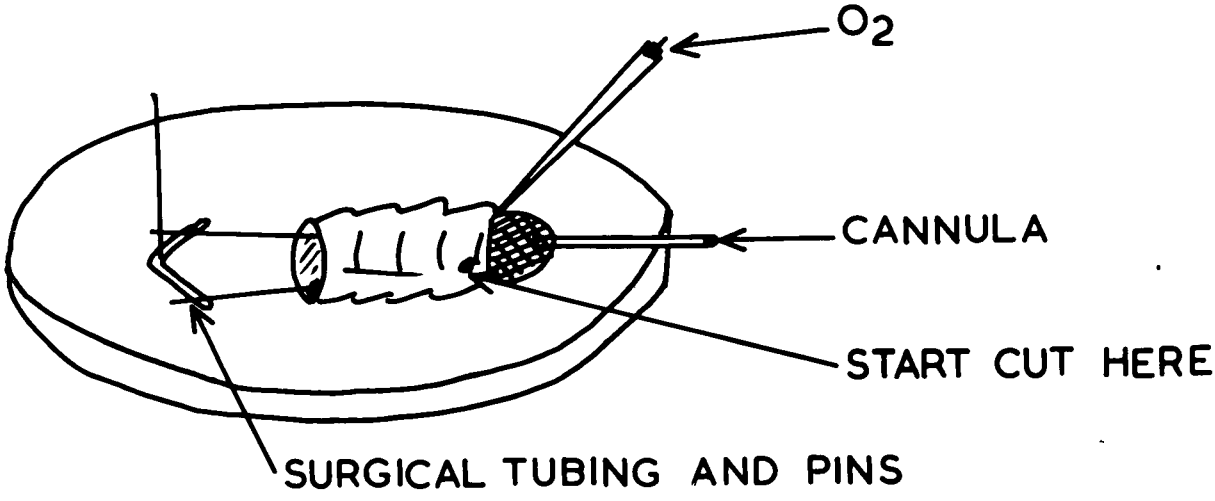
The initial and final samples of rectal fluid were stored under liquid paraffin, on wax-coated porcelain spot plates. The spot plates were easily prepared by pouring molten wax into the depressions, then quickly pouring the

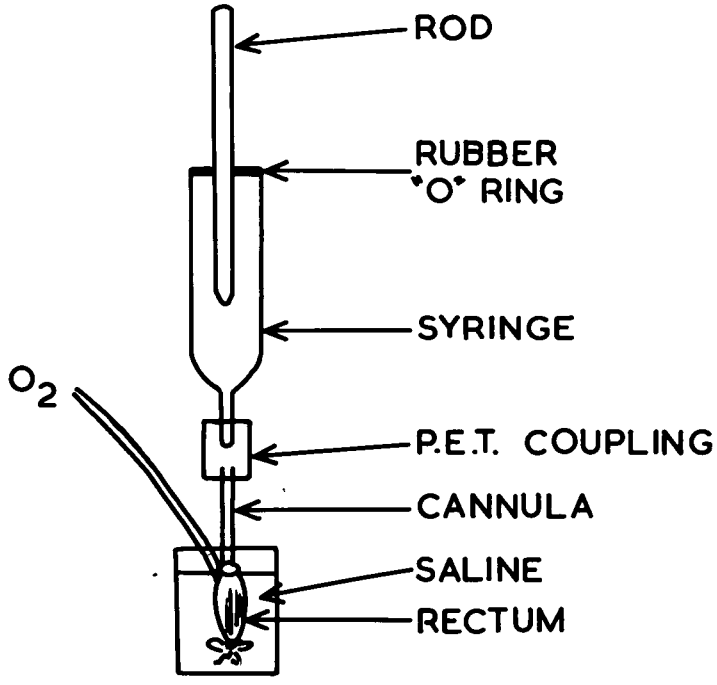
Fig. IV.

Diagram showing cannula in abdomen, prior to removal of rectum.

Fig. V.

Diagram showing injection apparatus with cannulated rectum *in vitro*.


O_2
CANNULA
START CUT HERE
SURGICAL TUBING AND PINS


ROD
RUBBER "O" RING
SYRINGE
O_2
P.E.T. COUPLING
CANNULA
SALINE
RECTUM

excess out. The spot plates with the samples were kept at $-10^{o}C$ if not analyzed immediately.

(e) Analyses of rectal fluid.

The osmotic pressure was determined by the cryoscopic method of Ramsay and Brown (1955), whereby the freezing point on aliquots of less than 1 ul were measured to within $\pm 0.01^{o}C$. Single determinations were made.

Chloride ion was measured by potentiometric titration of 1 ul aliquots of the sample with silver nitrate, using a Radiometer (model 25 ES) pH meter, after the method of Ramsay et al. (1955). Single determinations were made. The accuracy of this method is $\pm 1\%$.

Sodium was estimated on an E.E.L. or Unicam SP 900 flame photometer with an accuracy of $\pm 1\text{-}2\%$. The sample was prepared by diluting 1 ul aliquots in 4 ml of distilled water so that the deflection fell within the most sensitive range of the machine, 5 ppm for EEL, at which range there are no apparent interference effects from other ions, (Collins and Polkinhorne, 1952) . Several readings were made on each aliquot.

Potassium was estimated on the Unicam SP 900, by diluting 3 ul of sample with 6 ml of 500mM sodium chloride swamping solution. Duplicate samples were prepared and analyzed.

To measure pH, Radiometer microelectrodes with a sample working volume of 2 ul. were employed. Readings were

accurate to ±0.05 pH units on aliquots of 5 ul. of sample. The electrodes were coupled to a Radiometer (25 ES) pH meter.

Early attempts at measuring volume of rectal contents were made with P^{32} labelled radio-phosphate. Preliminary experiments indicated that this method was unsatisfactory due to loss of P^{32} activity. Subsequent experiments were carried out with inulin-C^{14}. Inulin does not penetrate the cuticular intima of the locust (Phillips, 1965). The injection fluid was prepared to contain roughly 1000 counts per minute per ul. Aliquots (3 ul.) were taken from the sample and added to 10 ml of scintillation fluid (Bray, 1960) and counted on a Nuclear Chicago liquid scintillation counter (Model 720), quench corrected by the channels ratio method. The extreme range of duplicate determinations on a rectal sample, including pipetting error and counting error, amounted to ±3%.

(f) Treatment of results

To compute the net volume change of rectal contents from the changes in the inulin-C^{14} activity, the following equation was used: $\%\ \text{water absorbed} = 100\left(1-\frac{C_i}{C_f}\right)$

where C_i is the initial concentration of the inulin as measured in counts per minute,

and C_f is the final concentration of inulin.

The concurrent movement of water must be considered when determining the net absorption of ions. This was

calculated as follows:

$$\% \text{ ion absorbed} = 100\left(1 - \frac{C_i\, I_f}{C_f\, I_i}\right)$$

where I_f is the final concentration of ion,

and I_i is the initial concentration of ion.

The differences in absorption between control and inhibitor-treated preparations were statistically analyzed for significance with a fortran computor program, using a randomized block design for analysis of variance, and testing with Dunett's Test as given by Steele and Torrie (1965). Correlations and regressions were tested using the Triangular Regression package available at the computing center.

(g) Measurement of transrectal potential.

The average net potential of 20 millivolts existing across the rectum, lumen positive (Phillips, 1964b), was considered to be a possible indicator of the performance of the in vitro preparation. This parameter was also required to determine which ions were actively transported.

Silver-silver chloride electrodes were fashioned as described by Phillips (1964b). These were inserted into bridges consisting of 3% agar solutions of either the injection fluid or the external saline. The electrodes were used to record from the internal and external fluids respectively. The preparation of the agar gels in this way avoids junction potentials between different solutions. All measurements of potential were made with a Radiometer (model 25 ES) pH meter.

The asymmetry potential was measured before and after each rectal potential by placing the tips of both electrodes in 3 Molar potassium chloride. The transrectal potential was corrected for this asymmetry potential.

Transrectal potentials were measured by withdrawing the rod from the injection apparatus (Fig. V) and inserting the drawn out internal electrode into the rectal fluid. The outer electrode was simply immersed in the external saline.

Results

(a) Preliminary experiments.

While developing the *in vitro* preparation, several variations of method were tested. Performance under various conditions was judged from ability of the preparation to concentrate the rectal content against a gradient. Thirty ul. of a sodium chloride solution (made hypertonic with sucrose, see methods) was injected into the rectum and left for 4 hours. The fluid remaining at the end of the experiment was analyzed for osmotic pressure and chloride. The results appear in Table 1.

Comparison of the freezing points of the injected solution and the final samples of rectal fluid clearly indicates that the *in vitro* preparation as described in the methods, and conditions 1 and 2 of Table 1, was concentrating the injected fluid against an osmotic gradient. Unlike the *in vivo* situation where chloride is actively absorbed from the lumen against a concentration gradient (Phillips, 1964), there is a large passive influx of chloride down a concentration gradient into the lumen of the *in vitro* rectum.

Removing the abdominal integument from around the rectum might damage the tissue by tearing some of the tracheoles out of the cells. Several experiments were done with the cuticle left in place (with the O_2 line into the hemocoel to ensure circulation), but this did not appear to change the results in terms of the concentrating ability of the organ. Since leaving the abdominal integument in

place made dissection easier and faster, and offered less chance of damage to the rectal sac, this procedure was adopted.

To determine whether the artificial saline was an adequate substitute for hemolymph, the performance of the preparation bathed in Schistocerca hemolymph was tested for comparison. Hemolymph was obtained from water-fed locusts by removal of a hind leg. The fluid could be collected in a pipette (0.4-0.5 ml per animal) and was used as a bathing media for the in vitro preparation. Since the results obtained did not indicate a substantially improved concentrating ability by the rectum and since frothing during oxygenation was a problem, the artificial saline (external solution) was used as the standard bathing solution in the remainder of the experiments.

Insects have a tracheal system for supplying oxygen directly to the tissues. It was considered that the bubbling of O_2 through the immersion media might not be adequate to supply oxygen to the luminal side of the rectum, since tracheoles penetrate deeply into the tissue. A crude "respirator" was attached to the cut end of the abdomen to apply a small positive and negative blood pressure cycle to the hemocoel. Hopefully this might simulate normal ventilation and assure proper oxygenation of the tissue via the normal route. The results did not indicate an improvement.

In one experiment, the external saline was not adjusted to pH 7.0. Complete inhibition of the concentrating

TABLE 1. SUMMARY OF PRELIMINARY EXPERIMENTS ON THE IN VITRO RECTUM

Condition	Freezing Point Depression (- Δ °C.)	Chloride Concentration (m. equiv./l.)
	I. Rectal Fluid at End of Experiment	
1. Abdominal cuticle completely removed leaving only rectal sac	1.29, 1.44, 1.23, 1.33	144, 137, 119
2. Abdominal cuticle left in place, O_2 bubbled into hemocoel	1.29, 1.26, 1.68, 1.48	172, 111, 133, 139
3. Abdominal cuticle left in place, connected to "respirator"	1.18, 1.76, 1.06, 1.91	146
4. Abdominal cuticle left in place, immersed in locust hemolymph (Δ °C .83)	1.28, 1.11	100, 125
5. Abdominal cuticle left in place, pH of external saline 5.5	.70, .75, .75, .82	61.5, 50.0
	II. Initial Values	
External saline	-0.75	125
Injected (internal) saline	-0.92	75

mechanism occurred, indicating a sensitivity to acid pH of the outer bathing solution.

To test that the observed concentration was due to rectal activity and not to evaporation within the small space of the injection apparatus, several preparations were made substituting polyethylene tubing in place of the rectal sac. The same procedures of injecting and sampling fluid were followed (see methods). The volume indicator showed a concentration change within the 3% counting error, while the osmotic pressure changes were less than 5 millios-moles/l. The experiment with external saline at pH 5.5 also confirms that the concentrating effect was due to rectal activity. Thus the in vitro preparation is capable of measureable metabolic activity over a 5 hour period.

(b) The effect of inhibitors at 10^{-3} Molar.

Inhibitors were used to test the relationship between ion movements, water movements, and metabolism. Potassium cyanide (KCN), an inhibitor of electron transport, was used to inhibit aerobic respiration. Ouabain is a specific inhibitor of membrane ATP'ase and the sodium-potassium pump, and was used to inhibit transport of these cations by the in vitro rectal sac.

Recta were dissected out into saline containing one of the inhibitors, or into the uninhibited control saline. A 30 ul. aliquot of the previously described hypertonic saline solution was injected within 5 minutes

of the operation and a 10 ul. sample withdrawn within 1 minute. The final sample (10 - 15 ul.) was removed after five hours. The initial and final samples were then analyzed for volume, osmotic pressure, sodium and chloride ions, and the net changes in rectal content of these parameters computed. In all, 12 experiments, each involving 3 preparations (1 control and 2 inhibited), were run. The mean net changes in rectal content appear in Table 2.

The control preparations show water absorption against an increasing osmotic gradient, since the osmotic pressure increases from 490 milliosmolar to 640 milliosmolar. The sodium is actively absorbed by the rectum against a concentration gradient. This uptake represents a decrease in initial concentration of about 9%. Chloride ion shows a passive flux into the lumen down an electrical and a concentration gradient, increasing the concentration by 3%.

The effects of the 10^{-3}M. ouabain and cyanide are not significant when compared to the control. Since these preparations had not been soaked in the inhibitor prior to injection of rectal fluid, the experiment was repeated in an identical manner except that the preparations were pretreated with inhibitor for one hour to allow additional time for diffusion to the active sites. In addition the effect of 10^{-3} Molar di-nitro-phenol (DNP), an uncoupler of oxidative phosphorylation from electron transport, was tested. Also, iodoacetic acid was added to the KCN solu-

TABLE 2 THE NET CHANGE IN RECTAL CONTENTS OVER A 4 HOUR PERIOD. A negative sign indicates net absorption or a decrease in rectal concentration and a positive sign an increase in rectal concentration.

Net Change in Rectal Contents	Control	KCN 10^{-3}M	Ouabain 10^{-3}M	$S_{\bar{d}}$
Volume (% of initial)	- 18.5	-10.9	-24.8	$\pm$ 3.4
Osmotic Pressure (m.osmole/l.)	+151	+91	+209	$\pm$56
Na (u. equiv.)	- 0.35	- 0.07	+ 0.25	$\pm$ .18
Cl (u. equiv.)	+ 0.06	+ 0.35	+ 0.30	$\pm$ .15

tion since it is known to inhibit anaerobic glycolysis, and should therefore show whether the preparation was dependent on anaerobic metabolism. Fifty ul of injection fluid were injected rather than 30 ul, allowing a larger volume of final sample and hence duplicate measurements of volume and sodium. In addition, potassium concentration was measured in duplicate.

The composition of the fluid prior to injection is given in Table 3, and immediately after injection and at the end of the five hour experiment in Table 4.

The net changes in the rectal volume, osmotic pressure, and sodium, potassium and chloride contents are presented in Table 5. These data are the means computed from 12 experiments, each involving four preparations (1 control and 3 inhibited).

The control preparation performed as described previously, pumping water and sodium out of the rectum against a concentration gradient, while the osmotic pressure gradient increased. Chloride and potassium entered the rectum passively, down a concentration gradient. Iodoacetate inhibits sodium transport and leads to a small passive influx of this ion into the lumen, yet water continues to be pumped out against a gradient. Ouabain has no significant effect on the activity of the preparation, as shown previously, although slight inhibition of the sodium absorption might have occurred since many of the measurements indicated a sodium flux into the rectum. There was much variation and this difference is not signi-

TABLE 3 INITIAL COMPOSITION OF EXTERNAL SALINE, AND INJECTION FLUID (MADE HYPERTONIC WITH SUCROSE), PRIOR TO INJECTION. INHIBITORS (10^{-3}M) ARE ADDED TO EXTERNAL SALINE.

Solution	O.P. m.osmoles/l.	Na m.equiv./l.	Cl m.equiv./l.	K m.equiv./l.
External Saline	376	105	125	4.8
Internal Saline	420	65	45	0

TABLE 4 RECTAL FLUID CONCENTRATIONS OF INITIAL (1 MINUTE) AND FINAL (AFTER 5 HOURS) SAMPLES, IN THE IN VITRO RECTUM DURING EXPERIMENT WITH 10^{-3}M INHIBITOR

		O.P. m.osmole/l.	Vol. C.P.M.	Na m.equiv./l.	K m.equiv./l.	Cl m.equiv./l.
Initial	Control	.366	152.7	63.6	0.7	47.4
	DNP	.375	152.6	62.9	1.6	50.1
	Ouab.	.377	156.2	63.3	1.8	50.8
	KCN+IAA	.380	157.6	66.2	1.5	51.3
	$S_{\bar{d}}$	±.01	± 4.0	± 2.0	±0.6	± 1.9
Final	Control	.508	208.0	79.1	6.9	78.2
	DNP	.462	137.0	75.6	6.5	70.5
	Ouab.	.501	190.6	79.7	4.7	72.9
	KCN+IAA	.573	205.2	112.4	7.1	86.7
	$S_{\bar{d}}$	±.028	± 10.1	± 13.0	±1.69	± 5.2

Analyses of variance shows the variation in initial O.P., Vol., and Na to be due to replication and not treatment.

TABLE 5. THE EFFECTS OF INHIBITORS AT 10^{-3} MOLAR CONCENTRATION ON IN VITRO RECTAL ABSORPTION OVER A PERIOD OF 5 HOURS, AFTER A PRE-INCUBATION OF ONE HOUR IN THE INHIBITOR.

A positive sign indicates a net increase in rectal content, a negative sign indicates a net decrease.

Significant differences between control and inhibited preparations are indicated by (**) for P=0.01 and (*) for P=.05

Net change in rectal content over 5 hours	Control	DNP	KCN+IAA	Ouabain	$S_{\bar{d}}$
Volume (% initial)	-24.4	+18.5**	-20.4	-14.6	±4.8
Osmotic Pressure (m.osmole/l.)	+141	+87	+193	+124	±40
Sodium (u.equiv.)	-0.155	+0.108*	+0.088*	+0.012	±0.005
Chloride (u.equiv.)	+0.047	+0.11*	+0.06	+.031	±0.02
Potassium (u.equiv.)	+0.175	+0.25	+0.186	+0.106	±0.03

ficant. DNP completely inhibited net water and sodium uptake, so that the direction of movement was into the rectum, the reverse direction from that in the control.

Calculation of the sodium concentration of the transported fluid in the control preparation shows the concentration of this fluid to be very low compared to the rectal fluid:

injected volume = 40 ul.

24.4% of 40 ul. = 10 ul. of water absorbed.

Since .16 u. equiv. sodium absorbed,

sodium concentration of transported

solution = $\frac{.16 \times 10^{-6} M}{10 \times 10^{-6} l}$ = 0.016 M

or 16 millimolar.

The initial tonicity of the rectal content was 420 milliosmolar or about 420 millimolar (slightly more due to activity coef.), while the apparent tonicity of the transrectal fluid was 16 millimolar. Clearly, the water movement is not dependent on the creation of an osmotic gradient by sodium transport.

(c) Effect of inhibitors at 10^{-2} Molar.

The ineffectiveness of ouabain, KCN, and KCN plus iodoacetate, at concentrations of 10^{-3} Molar, in inhibiting rectal activity could be due to lack of penetration from the saline to the active sites, or the rectal tissue is insensitive to these inhibitors. In either case it was felt that higher concentrations of

TABLE 6. INITIAL COMPOSITION OF OUTER SALINE AND INJECTION FLUID (MADE HYPERTONIC WITH SUCROSE), PRIOR TO INJECTION. INHIBITORS (10^{-2}M) WERE ADDED TO THE EXTERNAL SALINE.

Solution	O.P. m.osmole/l.	Na m.equiv./l.	Cl m.equiv./l.	pH
Outer saline	398	130	125	7.0
Internal saline	452	87	83	6.5

TABLE 7. MEAN RECTAL FLUID CONCENTRATIONS OF INITIAL (1 MINUTE) AND FINAL SAMPLES (AFTER 5 HOURS) IN THE IN VITRO RECTUM DURING EXPERIMENT WITH 10^{-2}M INHIBITORS

		O.P. $-\Delta^{\circ}C.$	Vol. C.P.M.	Na m.equiv./l.	Cl m.equiv./l.	pH
		$S_{\bar{d}}$=.014	$S_{\bar{d}}$=4.45	$S_{\bar{d}}$=2.33	$S_{\bar{d}}$=3.3	$S_{\bar{d}}$=0.067
Initial	Control	.838	237.5	86.5	82.7	4.142
	KCN	.831	230.2	84.8	85.1	4.156
	KCN+IAA	.816	232.8	82.8	84.8	4.271
	Ouabain	.844	245.6	83.3	82.3	4.093
Final	Control	1.113	289.2	92.7	114.9	4.048
	KCN	1.044	263.9	96.5	122.9	3.997
	KCN+IAA	.798	178.0	73.0	96.8	4.527
	Ouabain	.901	238.1	86.4	100.7	4.186
		$S_{\bar{d}}$=.071	$S_{\bar{d}}$=19.7	$S_{\bar{d}}$=7.9	$S_{\bar{d}}$=9.3	$S_{\bar{d}}$=0.536

TABLE 8. THE EFFECTS OF INHIBITORS AT CONCENTRATION OF 10^{-2} MOLAR ON IN VITRO RECTAL ABSORPTION OVER A PERIOD OF 5 HOURS. THE PREPARATIONS WERE PRE-INCUBATED FOR ONE HOUR IN THE INHIBITOR, PRIOR TO BEGINNING THE EXPERIMENT.

A positive sign indicates a net increase in rectal contents, a negative sign indicates a net decrease.

Significant differences between control and inhibitor treated preparations are indicated by (**) for p=0.01 and by (*) for p=.05.

Net change in rectal contents.	Control	KCN	KCN+IAA	Ouabain	$S_{\bar{d}}$
Volume (%)	-16	-9.7	+33**	+6.6*	±0.2
Osmotic Pressure (m.osmole/l.)	+151	+114	-9.7**	+31**	±67
Sodium (u. equiv.)	-0.25	+0.03	+0.37*	+0.31*	±0.25
Chloride (u. equiv.)	+0.40	+0.68	+1.22*	+0.78	±0.30

the inhibitor might cause inhibition of the *in vitro* rectum.

The experiments were carried out in the same manner as previously described. Thirty ul. of a hypertonic injection fluid having the composition shown in Table 6 and described in the methods, was injected into the rectum after an initial one hour incubation in the saline (control) or in the saline plus inhibitor. An initial 10 ul. sample was taken immediately and a final sample after 5 hours. The mean concentrations of volume indicator, sodium, chloride, total solute, and pH immediately after injection and after 5 hours, are given in Table 7. The initial variation is probably due to salts and water present in the rectum before injection which then becomes mixed with the injection fluid. None of this small variation is significant. Clearly, the control and inhibited preparations are starting at the same base level of ion and volume indicator, and the significant changes are due to action of the epithelium in this *in vitro* preparation. The net changes in rectal contents are given in Table 8.

The control *in vitro* rectum behaved as in the previous two experiments. KCN was again found to be ineffective in inhibiting rectal activity, relative to the control. The addition of iodoacetate to the KCN completely inhibits both the active sodium transport against a concentration gradient, and the active water

transport against an increasing osmotic gradient seen in the control. Water actually moves down an activity gradient into the rectum in large quantity, and the passive influx of chloride is significantly higher than in the control, when the rectum is inhibited with iodoacetate. Ouabain appears to inhibit the preparation to approximately the same degree as iodoacetate.

If the sodium concentration of the solution transported out of the control rectal sac is calculated as in the previous section, the fluid is again seen to be very hypotonic to the solution remaining in the rectum, confirming the earlier observation that sodium uptake by the rectum does not account for the water uptake.

(d) Statistical analyses of data; correlation and regression.

Individual preparations show considerable variation in the previous experiments. It was hoped that by taking advantage of this variability and regressing one parameter with another in the same preparation, some additional understanding of the interrelationships between water movements, ion fluxes and metabolism might be acquired.

The data for the 10^{-3} Molar, and 10^{-2} Molar experiments were tested for all possible regressions between the measured parameters. The significant regres-

TABLE 9. SIGNIFICANT CORRELATIONS DRAWN FROM THE EXPERIMENTS WITH IN VITRO RECTUM UNDER CONTROL AND INHIBITED CONDITIONS.

The correlation coefficient "r" indicates the goodness of fit of the regression line (if all points fell on line r=1.0)

The "F" is the symbol for the F test which assesses how much of the variation in Y can be accounted for by variation in X. A large F indicates good correlation.

The significance is expressed as probability values and indicated by (**) for p=0.01 and by (*) for p=0.05.

TABLE OF SIGNIFICANT CORRELATIONS AT 10^{-2} MOLAR CONCENTRATIONS OF INHIBITOR.

	Equation for regression	r	p	F	p
Osmotic Pressure on Volume					
Control	Y=.31 + .0023x	.196		.35	
KCN	Y=.125+(-.009x)	-.675	*	7.53	**
Ouabain	Y=.071+(-.002x)	-.663	*	7.05	*
KCN+IAA	Y=.069+(-.003x)	-.751	**	11.66	**
Volume on Chloride					
Control	Y=-23.13 + .42x	.747	**	11.37	**
KCN	Y=-16.9 + .27x	.393		1.65	
Ouabain	Y=-13.0 + .63x	.908	**	42.46	**
KCN+IAA	Y=-25.4 + 1.19x	.705	*	8.88	*+
Chloride on Sodium					
Control	Y=26.53 + 1.04x	.795	**	15.43	**
KCN	Y=26.1 + .79x	.831	**	20.01	**
Ouabain	Y=17.7 + 1.12x	.799	**	15.92	**
KCN+IAA	Y=40.35 + 0.63x	.649	*	6.58	*

TABLE OF SIGNIFICANT CORRELATIONS AT 10^{-3} MOLAR CONCENTRATIONS OF INHIBITOR.

		r	p	F	p
Osmotic Pressure on Volume					
Control	Y= -.33 + (-.007x)	-.67	*	8.09	*
24D	Y= .03 + .003x	.29	-	.95	-
Ouabain	Y= .05 + (-.005x)	-.794	**	17.1	**
KCN+IAA	Y= .14 + (-.003x)	-.63	*	6.43	*
Volume on Chloride			-		-
Control	Y=-21.1 + (-.13x)	-.25		.72	
24D	Y= 12.8 + .09x	.26		.75	
Ouabain	Y=-21.1 + .37x	.54		4.04	
KCN+IAA	Y=-36.9 + .49x	.93	**	61.3	**
Chloride on Sodium			--		--
Control	Y= 31.3 + .86x	.757	**	13.41	**
24D	Y= 45.2 + .76x	.30	--	.74	--
Ouabain	Y= 15.0 + .5x	.392		.81	
KCN+IAA	Y= 33.2 + .002x	.004		.0002	

sions appear in Table 9.

The volume change is negatively correlated with osmotic pressure; that is, the better the water pump performance, the greater the increase in osmotic pressure. Volume change is positively correlated with chloride, indicating that reduced water pump performance accompanies large passive chloride ion influx into the rectum. Sodium absorption is also positively correlated with chloride ion influx, i.e. large chloride influx accompanies low sodium transport or high sodium influx. Sodium does not appear to be correlated significantly with volume change. Correlations were not found in every case, as seen in Table 9. However, that they occurred at a 95% level of significance several times and that nearly all the correlations showed similar equations whether significant or not, appears to indicate that the interrelationships discussed above exist.

(e) Transrectal potential.

To evaluate which ion movements were active and which were passive in the previous experiments, the transrectal potential was followed over the same period of time (5 hours) and under the same conditions of control and inhibitor as in the experiments with 10^{-2} Molar inhibitor. The experimental procedure was the same as in previous experiments, the potential difference being measured when the initial and final samples would normally be taken. Some preparations were measured immediately upon dissection, but most were measured after one hour incubation. The

TABLE 10. THE MEAN TRANSRECTAL POTENTIAL AT THE BEGINNING AND END OF A DUPLICATE EXPERIMENT USING INHIBITORS AT 10^{-2}M. N.B. SIGN REFERS TO LUMEN RELATIVE TO HEMOCOEL. MEAN ± S.D. (NO. OF OBSERVATION)

Preparation	Potential Difference (Millivolts) At Injection Time	After 5 Hours
Control	17 ± 16 (12)	-2 ± 5 (9)
KCN 10^{-2}M	12 ± 8 (12)	-3 ± 6 (9)
KCN+IAA 10^{-2}M	15 ± 9 (12)	-1 ± 3 (9)
Ouabain 10^{-2}M	25 ± 17 (12)	-2 ± 8 (9)

results are shown in Fig. VI and Fig. VII. Table 10 gives the means and standard deviations at injection time and 5 hours later. The curves in the graphs are not statistically different so the inhibitors do not seem to be effecting the transrectal potential relative to the control.

(f) Measurements of rectal swelling.

To check that the volume change was truly indicative of transrectal transport of water, and not a tissue swelling, recta were weighed on a 50 mgm torsion balance before and after the in vitro experiments. The rectal sac was quickly dissected out in saline, blotted on filter paper and weighed. The very slow rate of weight change of the tissue while on the balance suggest, by extrapolation, that weight change was negligible during the interval between dissection and weighing. The initial average weight was 13.9 mgm (range 13.4 - 14.4 mgm). The final average weight was 12.1 mgm (range 10.6 - 14.0 mgm). None of the final weights exceeded the range of initial weights, so substantial swelling did not take place, and could not account for volume loss by the rectal contents, which averaged 10 mgm in the control preparations.

Fig. VI.

Graph showing the change in transrectal potential with time, as measured under conditions of experiments at inhibitor concentrations of 10^{-2} Molar.

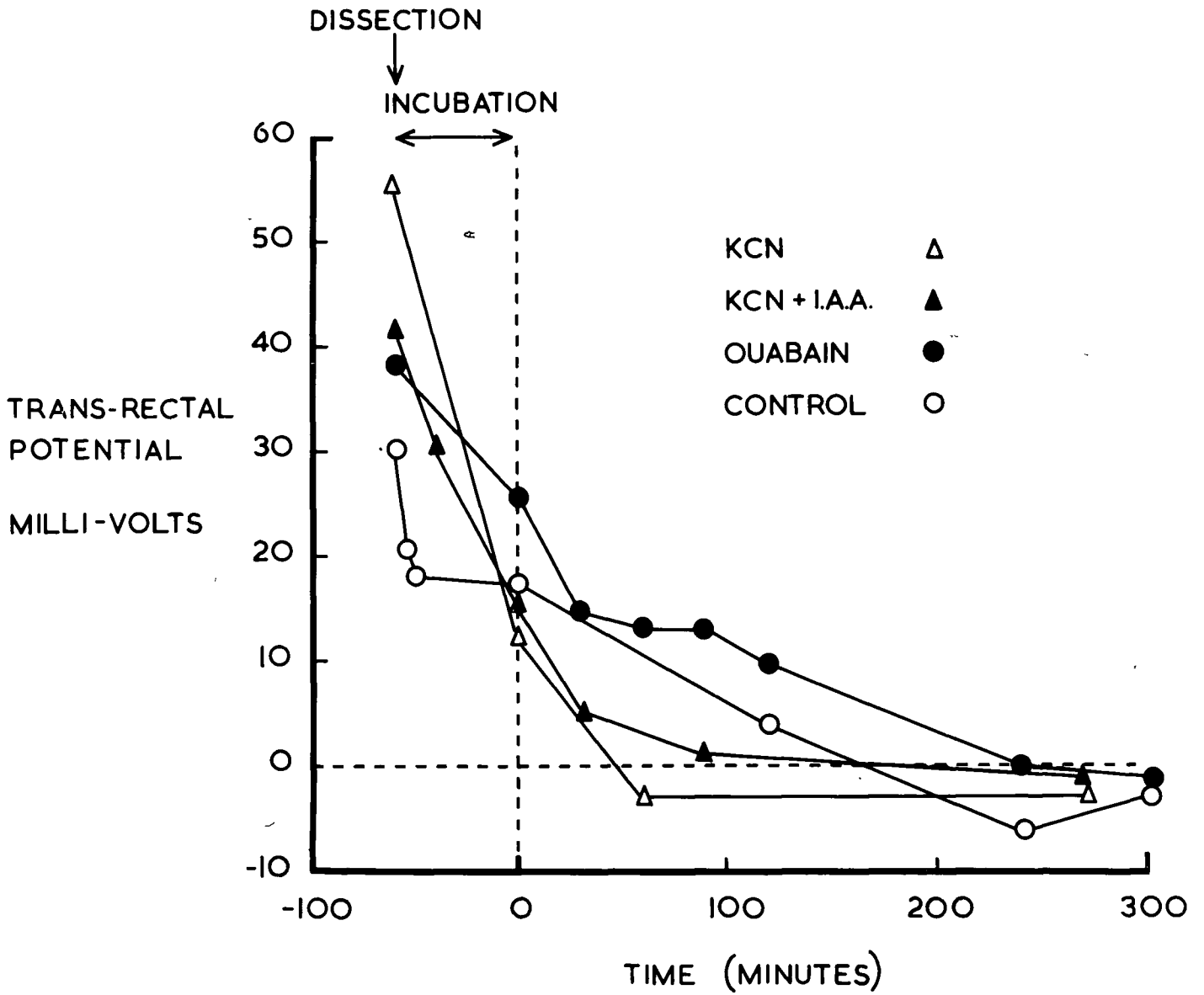
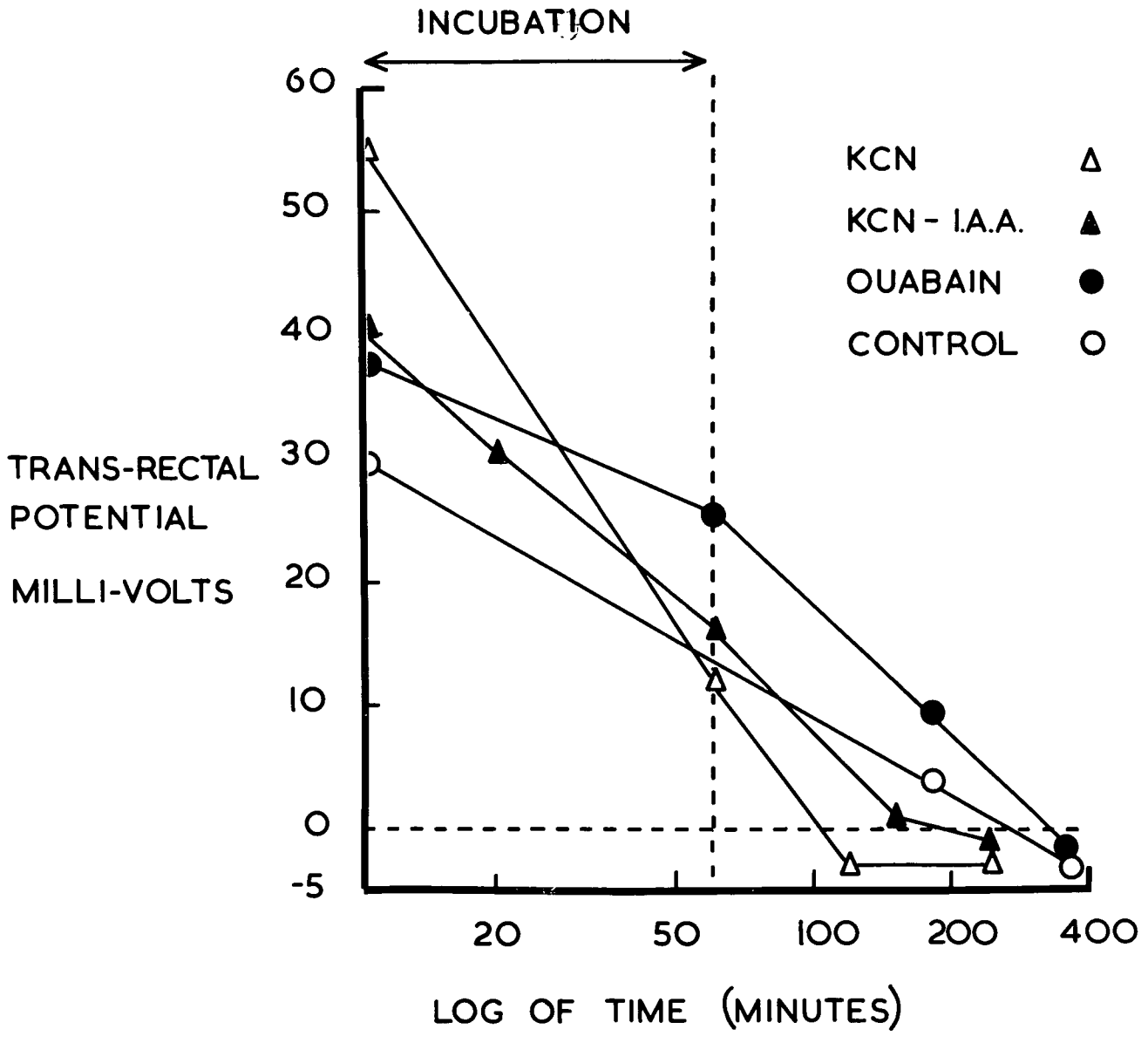

DISSECTION
INCUBATION
60
50
40
30
20
10
0
-10
TRANS-RECTAL
POTENTIAL
MILLI-VOLTS
KCN
KCN + I.A.A.
OUABAIN
CONTROL
-100
0
100
200
300
TIME (MINUTES)

Fig. VII.

Graph showing the change in transrectal potential with the $\log_{10}$ time, as measured under conditions of experiments at inhibitor concentrations of 10^{-2} Molar.


INCUBATION
60
50
40
30
20
10
0
-5
TRANS-RECTAL
POTENTIAL
MILLI-VOLTS
KCN
KCN - I.A.A.
OUABAIN
CONTROL
20
50
100
200
400
LOG OF TIME (MINUTES)

Discussion

Unlike the *in vivo* rectum of *Schistocerca*, the control *in vitro* preparation did not show any capability for chloride or potassium transport and only minimal sodium transport, out of the lumen. For example, *in vitro*, the final lumen concentrations of chloride and potassium are very close to the concentration of these ions in the bathing solution, while the *in vivo* preparation reduces the chloride in the rectum to 5% and the potassium to less than 2% of the hemolymph concentration. The *in vivo* rectum supports large ion concentration gradients, the *in vitro* rectum does not. The rates of ion movement in the control *in vitro* rectum are compared with the rates *in vivo* under similar conditions in Table 11. The *in vivo* rates are taken from Phillips (1961).

TABLE 11.

Ion	Mean net absorption rate (u equiv./hr./rectum)	
	In vivo	In vitro
Na	.16 ± .06	.05 ± .02
Cl	.20 ± .08	-.08 ± .04
K	.06 ± .01	-.035 ± .007
H_2O	6 ul./hr./rect.	2 ul./hr./rect.

Although these experiments were not done under exactly similar conditions (gradients) comparisons do indicate the radical differences between the two preparations. The

in vitro rectum preparation shows only about 30% of the activity of the *in vivo* situation, with respect to sodium and water. Clearly, caution should be used when extending *in vitro* results to the living animal; however, the preparation is useful in obtaining additional information about the mechanism of the observed water transport. *In vitro* and *in vivo* preparations of other animal tissue also have been shown to differ qualitatively. Frog skin (reviewed by Harris, 1960), for example, shows reduced rates of sodium and water transport when these are followed *in vitro*. Chloride transport has been demonstrated *in vivo* but not in the *in vitro* frog skin.

When comparison is made between the *in vitro* and *in vivo* preparations, consideration of the insect hormonal system should be made. A diuretic hormone secreted by ganglia in the head of *Schistocerca* has been shown by Higham, Hill and Gingell (1965). Maddrell (1962) discusses a diuretic hormone secreted by the abdominal ganglion of *Rhodnius*. What effect they might have on the rectum of *Schistocerca in vivo*, and the effect of their absence on the *in vitro* preparation, is unknown. The hormones could be regulating the rectal water and ion reabsorption.

The increase in osmotic pressure of the rectal fluid *in vitro* is due primarily to the absorption of water. This can be shown by the calculation that a 24% water loss can account for (.24 x 420 millios) about 100 of the 140 milliosmolar increase in osmotic pressure (control data

from Table 5). The remaining 40 milliosmolar increase is due to the influx of potassium, chloride, and probably some amino acids, into the rectum. The *in vitro* preparation clearly shows the ability to concentrate the solution it contains, by pumping water against an increasing osmotic gradient. Water transport can occur when potassium and chloride are moving into the rectum, and in the absence of an accompanying net efflux of sodium ion as seen in the experiment using iodoacetate and KCN at 10^{-3} Molar. In the latter experiment sodium shows a net movement into the rectum, while water is pumped out against the increasing osmotic gradient. Phillips (1961) demonstrated that no net transport of salts need accompany water transport. The present *in vitro* study has indicated that water movement against a gradient can take place even when the major ions are moving in the opposite direction. The water transport *in vitro* is in agreement with the observations of Phillips, and supports his findings by a different technique of volume indication, i.e. by use of Inulin-C^{14}.

In judging the activity of the *in vitro* rectum, consideration must be given to the net ion fluxes in terms of the electropotential gradient across the tissue. While the transrectal potential measured in the intact animal appears undiminished over 3 hours (Phillips, 1964), the potential shows a steady decline toward zero in the *in vitro* experiment. If the concentration gradient and the transrectal potential are considered together for each of sodium, potassium, and chloride ion, the electro-

chemical gradient is seen to favour chloride and potassium movement into the rectum throughout the experiment. This gradient is initially zero for sodium, but as the potential declines the concentration gradient favours movement of sodium into the rectum. Sodium absorption is therefore probably active, while the movements of potassium and chloride into the lumen are passive.

The independence of water movement from accompanying salt movement is substantiated by the calculation of the osmotic pressure of the transported fluid, which has been shown to be very hypotonic to the rectal fluid. In all vertebrate systems which have been studied, such as the frog skin (Ussing, 1960) and fish gall-bladder (Diamond, 1963), water is moved as an isosmotic or hyperosmotic solution, i.e. salt transport sets up a local osmotic pressure gradient. The independence of water transport from net salt transport appears to be different from any vertebrate system so far investigated.

The possibility that the volume decrease was not due to active rectal absorption of water, but rather to swelling of the rectal tissue was considered. The observed increase in rectal fluid potassium and decrease in sodium ion is the type of exchange one might expect if a cellular sodium-potassium pump at the luminal border (extruding sodium, accumulating potassium), were inhibited. Such an exchange would lead to some cellular swelling (Tosteson and Hoffman, 1960). If the <u>in vitro</u> preparation

were not being properly oxygenated, the reduced activity of such a Na-K pump would be expected to result from reduced energy supply. The amount of swelling necessary to account for the 24% volume reduction (control in 10^{-3}M experiments) or 10 ul, would amount to an increase of 10 mgm., or a doubling of rectal weight. This was not found. The volume loss is due to transrectal transport.

The performance of the in vitro rectum can be interpreted in terms of the hypothetical scheme (Fig. VIII) for cellular organization of rectal transport as suggested by Phillips (1965), if two assumptions are made. One assumption is that the rectal tissue was not being adequately oxygenated by diffusion from the external saline, an assumption supported by the observation that KCN 10^{-2}M had no measurable effect on the in vitro rectal activity.

The second assumption is that the lack of oxygen is most acute at the luminal border, having the dense population of mitochondria and being farthest from the oxygen source. If the metabolic transport activity at this membrane were generally inhibited, it can be seen that the passive movements would predominate and chloride and potassium would diffuse into the rectal lumen from the cells and hemocoel. Since there is evidence (Phillips, 1961) that the transrectal potential is a potassium diffusion potential, the diffusion of the potassium ion out of the rectal cells on the luminal

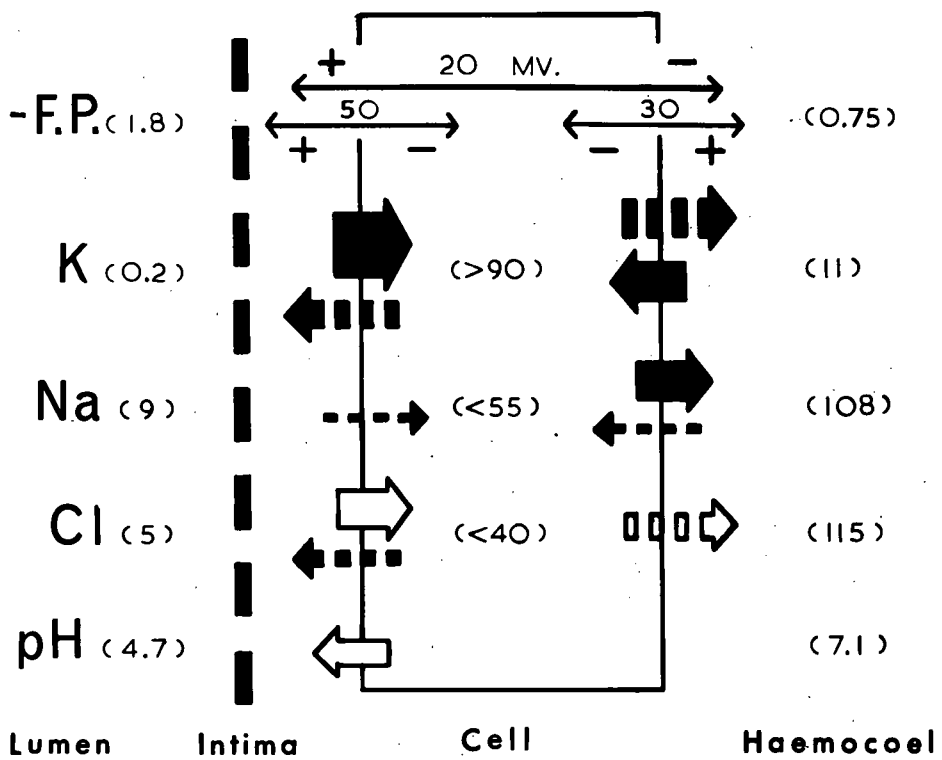
Fig. VIII.

Scheme of Phillips (1965) for cellular arrangement of mechanisms for rectal transport of ions, as indicated by *in vivo* observations.

The scheme indicates that active net ion movement is the result not only of an active transport component, (solid arrows), but also a passive or back diffusion component (broken arrows).

An increase in permeability, increasing the passive component, would have the same net result as a decrease in the active pumping mechanism.

Figures in brackets indicate ionic concentrations in m.equiv./l., pH units, or freezing-point depression (-F.P.) in ^{o}C. Unbracketed values indicate electropotential differences in millivolts.


+
20 MV.
−
-F.P. (1.8)
50
30
(0.75)
+
−
−
+
K (0.2)
(>90)
(11)
Na (9)
(<55)
(108)
Cl (5)
(<40)
(115)
pH (4.7)
(7.1)
Lumen
Intima
Cell
Haemocoel

side would result in a logarithmic decline of the potential across this membrane as the potassium concentration gradient decreases, so that the transrectal potential would approach zero millivolts.

Provided the hemocoel membrane functions as proposed in the scheme, while the lumen membrane is disrupted as just described, sodium ion could be expected to move passively from the lumen into the cells and probably be pumped into the hemocoel, explaining the slight uptake of sodium by the control preparations; however, sodium uptake might not be transrectal. On the other hand when inhibitors are applied to the hemocoel side, the sodium pump would be expected to be inhibited (metabolically or directly) and sodium would then move from the hemocoel to the lumen of the rectum, as was observed.

The correlations presented in Table 9 are consistent with the hypothesis presented thus far, in relation to the scheme in Fig. VIII. The positive correlation between volume change and chloride ion implies that when the membrane is working well, (i.e. when there is an adequate energy supply for transport mechanisms in general), the volume of rectal fluid at the end of the experiment is low, indicating good water transport, and the influx of chloride is low since residual chloride pump activity reduces net influx of chloride. A general variation in membrane transport activity could also explain the correlation of sodium and chloride. By reducing

the active component of ion movement for both ions, a correlation of low sodium transport and high chloride influx would result. The volume would be expected to correlate negatively with osmotic pressure since the osmotic pressure increases as the water is pumped out and the salts are left in the lumen of the rectum, although the correlation is not necessarily exact because of the net influx of solute.

Whether variation in net ion influx is due to inhibited pump activity as just described or to general changes in the passive permeability of the membranes is not known. Both suggestions fit all data equally well. An obvious test for a passive permeability increase between control and inhibited rectum *in vitro* could be to study ionic fluxes by placing labelled sodium and chloride on the serosal side of the rectum and measuring the rate of appearance of activity in the lumen.

Cyanide inhibits oxidative phosphorylation by binding irreversibly with cytochrome oxidase (reviewed by Harper, 1965). As a result, energy cannot be trapped in the form of high energy phosphate bonds, since the aerobic respiratory pathways are blocked. Vertebrate preparations such as fish gall-bladder are inhibited by concentrations of 10^{-4} Molar cyanide (Diamond, 1962). Extrusion of sodium ion by squid nerve is inhibited by 10^{-3} M. cyanide (Caldwell et al., 1960). The *in vitro* insect tissue used in the present experiments was

insensitive to cyanide at 10^{-2} Molar, relative to a control preparation. This insensitivity to the inhibitor at such high concentrations could be due to either of the following two suggestions. The cyanide is not getting to the site of inhibition or the cells in vitro are not utilizing cyanide-sensitive aerobic respiration. Since cyanide is a small ion which penetrates membranes readily, it is difficult to accept the first suggestion, especially since iodoacetate appears to be getting into the cells. The conclusion is that the in vitro preparation is not using aerobic respiration, possibly due to inadequate tissue oxygenation as previously discussed. It is seen from the electron micrographs in the section on structure that tracheoles penetrate the rectal cells almost to the lumen. Bubbling O_2 in the external saline does not appear to duplicate this system of delivering O_2 to the tissue. The in vitro rectum appears to be depending on anaerobic glycolysis as a supply of energy.

The addition of iodoacetic acid to the in vitro preparation completely inhibits water and sodium transport out of the rectum, and leads to passive influx. Iodoacetic acid is known to inhibit the enzyme glyceraldehyde-3-phosphate dehydrogenase, thus inhibiting glycolytic formation of ATP (Harper, 1965). The inhibition of the active mechanisms by iodoacetate therefore supports the assumption that the in vitro rectum preparation is depending upon anaerobic metabolism.

The previous discussion has indicated that the *in vitro* rectum is functioning anaerobically. It is therefore surprising that the inhibitor DNP(10^{-3} Molar) should have such a marked inhibitory effect upon water and sodium transport. DNP typically acts to uncouple oxidative phosphorylation from electron transport system but is ineffective in preventing glycolytic production of ATP. (reviewed by Harper, 1965).

A similar anomaly has been observed for the turtle bladder. Bricker and Klahr (1966) showed that DNP (10^{-5}M) inhibited anaerobic sodium transport in this tissue. These workers demonstrated biochemically that the glycolytic pathway was being completed. Inhibition of ATP synthesis was not the cause of the inhibited sodium transport. Also, ATP stores within the tissue were only reduced slightly. (DNP stimulates ATP'ase activity; Quastell, 1964). One of their hypotheses was that a high energy intermediate was involved between ATP and the sodium pump, and that this was being uncoupled from the pump by DNP. Whether a similar hypothesis may apply for DNP action on the *in vitro* rectum of *Schistocerca* is unknown. One should first demonstrate that glycolysis is not inhibited.

Other possibilities are that DNP is inhibiting the sodium and water pumps by directly affecting the transport mechanisms, or by changing the membrane permeability, rather than by inhibiting the energy source.

Ouabain is a cardiac glycoside and is a specific inhibitor of membrane ATP'ase. The compound prevents accumulation of potassium and extrusion of sodium by red blood cells, while not effecting glycolysis (Kahn, 1961). Since the inhibitory action is antagonized by high concentrations of external potassium, it is believed that ouabain is competing for the same membrane site as potassium. That this site is membrane ATP'ase is suggested from the comparison of the concentration of ouabain required to inhibit ATP'ase with the concentration required to inhibit the sodium-potassium pump. A steroid molecule, ouabain is believed to attach to the ATP'ase by the 23rd carbon and may inactivate the site simply by its large size (Repke, 1965). Ouabain blocks uphill transport of sodium-potassium in many tissues such as vertebrate kidney, skin, lens, red blood cell, thyroid, nerve, muscle, and salivary gland (Kahn, 1961), and such invertebrate tissue as crab nerve (Skou, 1957). It also changes the permeability of frog skin to water and chloride (MacRobbie and Ussing, 1961), blocks chloride transport in intestine (Cooperstain, 1959), and blocks sodium-dependent transport of non-electrolytes such as sugars and amino acids in intestine (Csaky, 1963).

According to Csaky, ouabain normally inhibits at concentrations of 10^{-7} - 10^{-5} Molar. Insect preparations have been found quite insensitive to ouabain.

Haskell, Clemons and Harvey (1965), and Maddrell (1966) found sodium transport in midgut and malpighian tubules to be unaffected by ouabain at concentrations of 10^{-3} Molar. Treherne (1966), however, has demonstrated that ouabain at 10^{-4} Molar inhibits sodium extrusion from insect nerve.

In the present experiments with Schistocerca rectum, 10^{-3} Molar ouabain did not significantly inhibit sodium transport, and had no apparent effect upon water transport out of the rectum. At 10^{-2} Molar both transports were inhibited. The high ouabain concentrations may have been necessary for several reasons.

Repke (1965) has shown that ouabain is only 1/5 as effective an inhibitor of ATP'ase at pH 5, when compared to pH 7. It is known that rectal cells of Schistocerca secrete hydrogen ion into the lumen (Phillips, 1964). The intracellular pH is unknown. If the intracellular pH were acid, and if the ouabain were acting at the luminal membrane (it was applied to the serosal side), then a high concentration of the inhibitor would be required to counteract the effect of the acid pH. The large molecular size (M.W.=729) and the fact that it has never been shown that ouabain enters cells (Csaky, 1963) makes this explanation unlikely.

A second explanation for the high concentration

required, involves the permeability of the rectal tissue to ouabain. From the electron micrographs, one sees that the large molecule would have to diffuse through a layer of muscle and probably a layer of secondary cells, as well as two thick basement membranes, to get to its site of inhibitory action, assuming inhibition of the columnar cells. Clearly, a high saline concentration of ouabain increases the likelihood of the molecule arriving at its target by diffusion. If a diffusion barrier to ouabain is present, then the high concentration of 10^{-2} Molar may be required to reach an inhibiting concentration of 10^{-7} - 10^{-5} Molar at the active sites.

Supporting the low permeability of insect tissue, Larsen et al. (1966) found curare-like effects, similar to those for vertebrate neuro-muscular junctions, could be elicited only at high concentration of the inhibitor (10^{-2}M.). The effects were found reversible.

A third possibility is that insect tissue is generally insensitive to ouabain, though Treherne's work puts this in doubt. Progesterone and testosterone antagonize the action of ouabain (Csaky, 1963). Perhaps the insect tissue is protected from ouabain by insect steroids in the tissue and hemolymph.

Whether ouabain at 10^{-2} Molar inhibits rectal water transport by inhibiting a sodium-potassium pump

(linking the water transport to ion transport) or by causing general metabolic arrest or permeability change has not been determined. The separation of these alternatives would require considerably more experimentation. At present no known adverse metabolic effects occur at high ouabain concentrations, but from the work of Tosteson (1964) on volume regulation and the maintenance of cell milieu, if the sodium-potassium pump were inhibited at 10^{-2} Molar ouabain concentrations, one would expect the loss of potassium and increase in sodium to lead to an increase in osmotic pressure. This would result in increased water influx, increasing cell volume, and causing a general permeability increase. Conceivably, this could result in an interruption of cell metabolism.

The interpretation of the results with ouabain must be approached with caution since the effects of the inhibitor at this high concentration are unknown. The fact that it is a steroid and that steroids are lipid soluble, suggests that ouabain could cause a change in membrane permeability. Whether the inhibition of the water pump is due to inhibited ion transport or increased permeability to water and back diffusion of ions, can not be stated.

The observation that uphill water transport can occur out of the rectum when the salt movements are all into the rectum, makes a very strong case for active transport of water in the absence of net accompanying

movement of salts. The three schemes presented in the introduction suggested how a recycling local ion pump might explain the water transport in the absence of salt transport. If the assumption is made that this pump is the sodium-exchange pump, then the observation that ouabain, 10^{-2} Molar, inhibits water transport supports the three schemes' dependence on a local ion transport. However, with 10^{-3} Molar potassium cyanide and iodoacetate, sodium transport appears to be eliminated, yet water movement against an increasing gradient is still observed. This evidence tends to separate the water pump from a dependence upon ion transport.

A study of ionic fluxes and changes in tissue ionic concentrations during absorption in vitro might provide more insight into the water transport. The results, however, show the water movement to be dependent upon cell metabolism, a fact hitherto not clearly demonstrated.

SUMMARY.

1. The general ultrastructure of the columnar epithelium and secondary cells of the rectal pad is described.
2. This tissue appears to consist of two cell layers (specialized for active transcellular transport) in series, rather than a single layer as previously supposed.
3. Ultrastructural observations to date are consistent with all three hypotheses of water transport suggested in this thesis.
4. An *in vitro* preparation of the locust rectum was developed and the effects of various metabolic inhibitors on water and ion absorption from the preparation were studied, using inulin-C^{14} as a volume indicator.
5. The *in vitro* rectum retains the capacity to absorb sodium and water against an increasing osmotic pressure gradient but, unlike the *in vivo* rectum, does not maintain the normal transrectal potential or the active absorption of chloride and potassium ions.
6. Water absorption was observed when net movement of all monovalent ions was into the lumen, as in the presence of potassium cyanide (10^{-3}M) plus iodoacetate (10^{-3}M.).
7. Water and sodium transport *in vitro* are probably dependent on anaerobic respiration and are completely inhibited by iodoacetate (10^{-2}M.), ouabain (10^{-2}M.) and dinitrophenol (10^{-3}M.) but unaffected by potassium

cyanide (10^{-2} or 10^{-3}M.) or by lower concentrations of ouabain (10^{-3}M.).

8. The water absorption from the <u>in vitro</u> rectum is a transrectal movement and is not due to tissue swelling.

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