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DIRECT MICROPROBE EVIDENCE OF LOCAL CONCENTRATION GRADIENTS AND RECYCLING OF ELECTROLYTES DURING FLUID ABSORPTION IN THE RECTAL PAPILLAE OF *CALLIPHORA*

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SUMMARY

1. The concentrations of sodium, potassium and chloride and dry mass were measured by electron-probe X-ray micro-analysis in 1 μm thick frozen-hydrated sections from *Calliphora* rectum in 5 different states of absorptive function.

2. In all cases the average concentrations of sodium + potassium + chloride was consistently higher in the fluid in the lateral intercellular spaces than in the cytoplasm, the average ratio being 2:1 in water-fed flies and higher in water-deprived flies.

3. The highest concentration of electrolytes was found in the extracellular channel of complex lateral membrane stacks, which is consistent with the histochemical localization of major cation pumps at these sites (Berridge & Gupta, 1968). This concentration exceeded the electrolyte concentration in other tissue compartments by some 80 m-equiv/l H_2O in water-fed flies and about 700 m-equiv/l H_2O in water-deprived flies. The potassium and sodium concentration ratio of this extracellular fluid was nearly 1:1 in water-fed flies, 3:1 in water-deprived flies with KCl in the rectal lumen, and 0·5:1 with NaCl in the rectal lumen.

4. Results suggest that the extracellular fluid is generated in membrane infoldings along the intercellular channels, and that this fluid gains water and sodium, but loses a variable amount of potassium and chloride, as it passes to the haemolymph, thus supporting the idea of local osmosis and ion recycling.

INTRODUCTION

The study of electrolyte metabolism at the cell level ultimately requires methods for determining the concentrations of electrolytes in different parts of the cell and its extracellular surroundings. By electron-probe X-ray microanalysis (microprobe) it is possible to measure the concentrations of elements in selected microvolumes of a sample (Hall, 1971). Conventional fixation and dehydration procedures for electron microscopy alter membrane permeability (Morel, Baker & Wayland, 1971; Vassar

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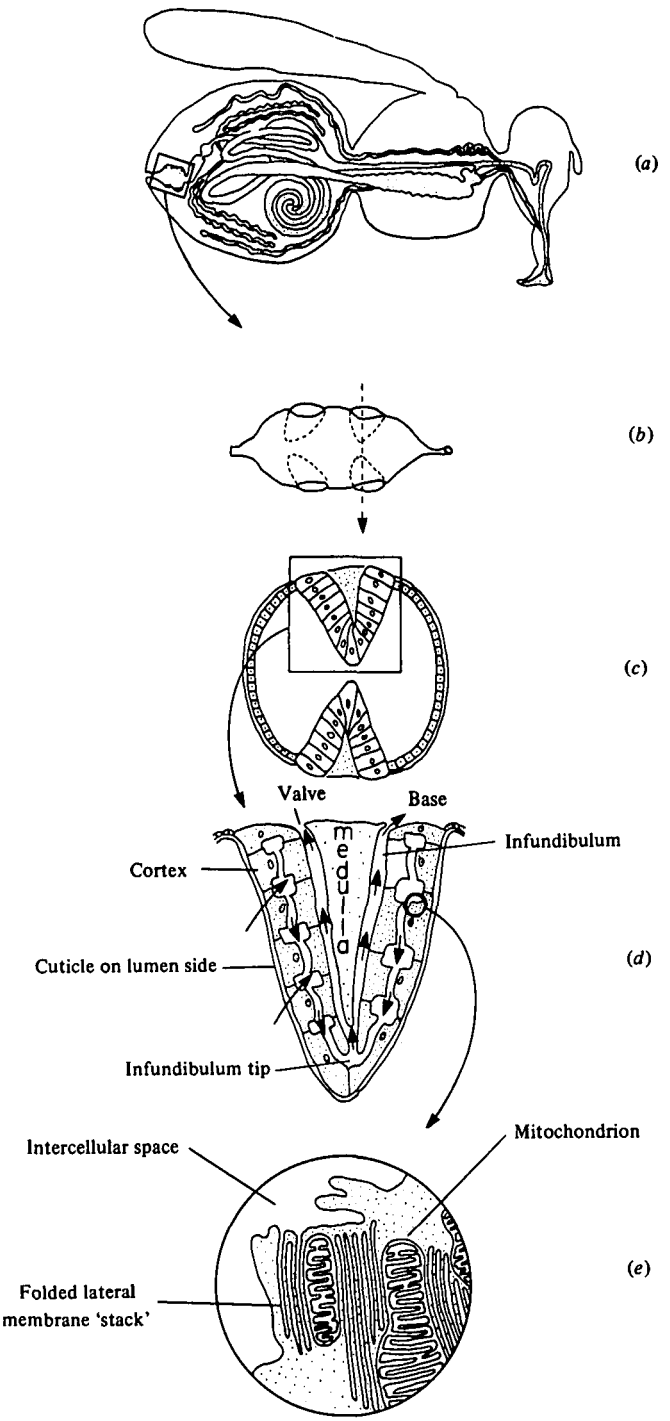
et al. 1972), leading to rapid redistribution of solutes and dissipation of concentration gradients (Hodson & Marshall, 1970; Porter, 1961; Schoenberg *et al.* 1973). In the present study of the local osmosis model of fluid transport, these problems are avoided by using frozen sections of unfixed tissues in the procedure developed in this laboratory (Gupta, 1976; Gupta, Hall & Moreton, 1977*a*; Gupta & Hall, 1978, 1979; Hall, 1979*b*). This model has been applied widely to both secretory and absorptive tissues in animals (Gupta & Berridge, 1966*a*; Kaye *et al.* 1966; Tormey & Diamond, 1967; Berridge, 1968; Berridge & Oschman, 1969, 1972; Diamond & Bossert, 1968; Oschman & Berridge, 1970) and to water movements in plants (Anderson, 1974). The basic hypothesis is that electrolytes are actively transported into narrow compartments such as intercellular spaces, establishing a local increase in the osmotic pressure. Such hypertonic interspaces then draw water by osmosis, either via the cytoplasm or paracellularly through cell junctions (Gupta & Hall, 1979).

It is not yet possible to test the local osmosis model by measurements of osmotic and ionic activities in complex tissues because such measurements cannot be made at sufficiently precise locations *in situ*. In most recent mathematical models for explaining isosmotic fluid transport, it has been proposed that the anticipated osmotic gradients in the relevant interspaces are either so small that they cannot be accurately measured (Machen & Diamond, 1969; Diamond, 1977, 1978, 1979; Sackin & Boulpaep, 1975; Schafer, 1979) or they do not exist (Hill, 1975, 1977). But microprobe studies have now shown that the fluid in the relevant interspaces of tissues such as *Rhodnius* Malpighian tubules (Gupta *et al.* 1976), *Calliphora* salivary glands (Gupta *et al.* 1978*a*), rabbit ileum (Gupta, Hall & Naftalin, 1978) has a much higher concentration of electrolytes than in other tissue compartments. This may mean that the osmolality of the fluid in the interspaces is 50–100 m-osmole higher than that of the bathing fluid (300–350 m-osmole), although the exact magnitude of interspace hyperosmolality in these measurements remains uncertain (Gupta & Hall, 1979) because the microprobe measures only total electrolyte content and not the osmotic activity.

In contrast to tissues which transport isosmotic fluids, the insect rectum is specialized for regulated water absorption against very large osmotic gradients (Ramsay, 1971). For a local osmotic mechanism to operate, the osmotic concentrations within the relevant interspaces in rectal epithelium should be much larger and therefore easier to measure. Indeed, the insect rectum is the only tissue from which intercellular fluid has been collected and its osmotic pressure and Na and K concentration determined (Wall, Oschman & Schmidt-Nielsen, 1970).

Fig. 1. Summary of morphology of blowfly rectal papilla. (*a*) Diagrammatic view of alimentary canal and associated structure. (*b*) Diagram of rectum showing four cone-shaped rectal papillae protruding into rectal lumen. (*c*) Cross-section view of rectum through two rectal papillae. (*d*) Each rectal papilla consists of an inner medulla (composed primarily of connective tissue, tracheae and neurosecretory nerve terminals) and a cortex, facing the lumen, composed of transporting epithelial cells. Cortical epithelium contains an elaborate labyrinth of intercellular spaces. Fluid movement (shown by arrows) is from the lumen into intercellular spaces, towards the apex of a papilla, then basally through the infundibulum, ultimately emerging into the body cavity via an infundibular valve (Graham-Smith, 1934; Gupta & Berridge, 1966*a*; Berridge & Gupta, 1967). (*e*) Lateral membranes of rectal papilla cells are folded to form 'stacks' that are closely associated with mitochondria and are rich in Mg^{2+}-activated ATPase activity (cf. Fig. 2). These membrane stacks seem to be primary sites of solute transport from cells into intercellular spaces (Berridge & Gupta, 1967).


(a)
(b)
(c)
Valve
Base
Infundibulum
Cortex
medulla
Cuticle on lumen side
(d)
Infundibulum tip
Intercellular space
Mitochondrion
(e)
Folded lateral
membrane 'stack'

In the blowfly, as in many insects, water absorption by the rectum is performed by specialized cells in rectal papillae. The gross morphology and fine structure of blowfly rectum have been described elsewhere (Graham-Smith, 1934; Berridge & Gupta, 1967; Gupta & Berridge, 1966*a*, *b*) and are summarized in Fig. 1. Each papilla is a cone-shaped structure (Fig. 1 *a–d*) consisting of an inner medulla (composed primarily of connective tissue, tracheae, and neurosecretory nerve terminals) and a cortex, facing the lumen, composed of transporting epithelial cells (Fig. 1*c*, *d*). The cortical epithelium contains an elaborate labyrinth of intercellular spaces (Figs. 1 *d*, *e*; 2).

The membranes of the cortical epithelial cells are piled together to form stacks that are closely associated with mitochondria (Gupta & Berridge, 1966*a*; Berridge & Gupta, 1967). The membranes of the stacks are rich in Mg^{2+}-activated ATPase activity (Berridge & Gupta, 1968). It was suggested that these membrane stacks are the primary sites of solute transport from the cells into the intercellular spaces. Water was thought to move by osmosis from the lumen into the intercellular spaces, and then to follow the intercellular route indicated by dye-injection (Graham-Smith, 1934): toward the apex of the papilla and then basally through an 'infundibulum', ultimately emerging into the body cavity or haemocoel via an infundibular 'valve'. It was also proposed that as the primary absorbate moves through the channel system, solutes, especially potassium, could be reabsorbed from it by the cells and recycled (Berridge & Gupta, 1967).

We have applied our microprobe techniques to measure the concentrations of Na, K and Cl within different intracellular and extracellular compartments of *Calliphora* rectal papillae. The results are entirely consistent with the above proposed mechanism of ion and water absorption.

MATERIALS AND METHODS

Blowflies (*Calliphora erythrocephala*) were reared on sugar and pig's heart, with water available. Recta I and II were from these water-fed animals. Rectum III was from water-deprived flies, that had no water for 24 h. Other recta from water-deprived animals were injected with solutions of known composition. This was accomplished by dissecting open the abdomen to expose the rectum and injecting a few microlitres of solution into the rectal lumen via a cannula inserted into the anus. The injected solutions were: 100 mM KCl + 50 mM NaCl + 20% (w/v) dextran (m.w. about 237,000 daltons) (rectum IV); 100 mM KCl + 100 mM sucrose + 20% dextran (rectum V); or 100 mM NaCl + 100 mM sucrose + 20% dextran (rectum VI). The concentration of each fluid (less dextran) was 300 m-osmole, because this is believed to be the concentration of the fluid from the colon entering the rectum under all conditions (Phillips, 1969).

The animals were anaesthetized by cooling them in a freezer for about 3 minutes. The rectum was then dissected out and placed in a small drop of *Calliphora* Ringer solution (mM: Na, 132; K, 20; Ca, 2; Tris, 10; Cl, 158; phosphate, 4; malate, 2·7; glutamate, 2·7; glucose, 10; pH 7·2; Gupta *et al.* 1978*a*) in a depression on the end of a copper pin about 1 × 8 mm. Like the injection solutions mentioned above, the Ringer contained 20% (w/v) high-molecular-weight dextran in order to reduce ice

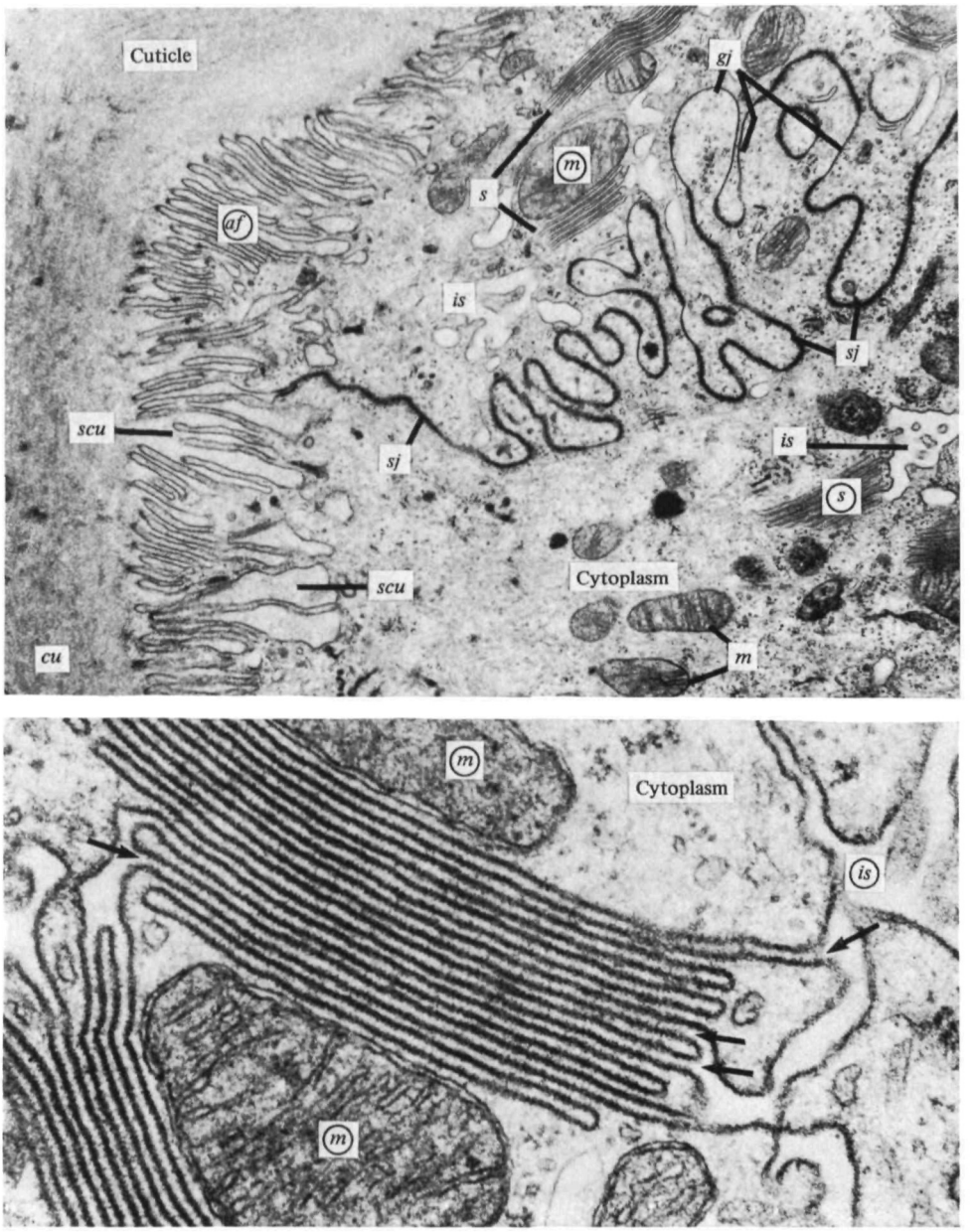


Fig. 2. Conventional transmission electron micrographs of stained Araldite sections of *Calliphora* rectal papillae, showing the complexity of structure (cf. Gupta & Berridge, 1966*a*, *b*; Berridge & Gupta, 1967; Lane, 1979). *Top.* A low magnification field from the apical part of the cortical cells (cf. Fig. 5) to show cuticle (*cu*), apical folds (*af*) with subcuticular spaces (*scu*), septate junctions (*sj*) incorporating gap junctions (*gj*), mitochondria (*m*), intercellular spaces (*is*) and stacks (*s*). For functional variations in structural geometry see Berridge & Gupta (1967). × 14000. *Bottom.* Details of a membrane stack to show membrane folds, mitochondria (*m*), and narrow extracellular channels opening (→) into intercellular spaces (*is*). × 28000.

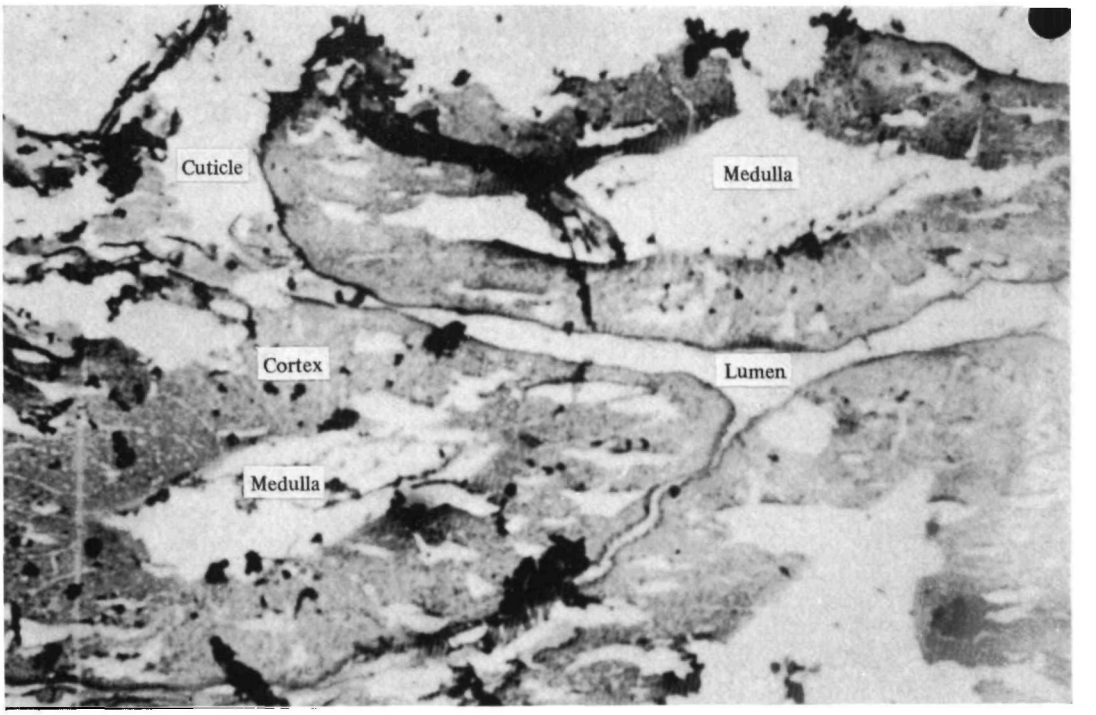
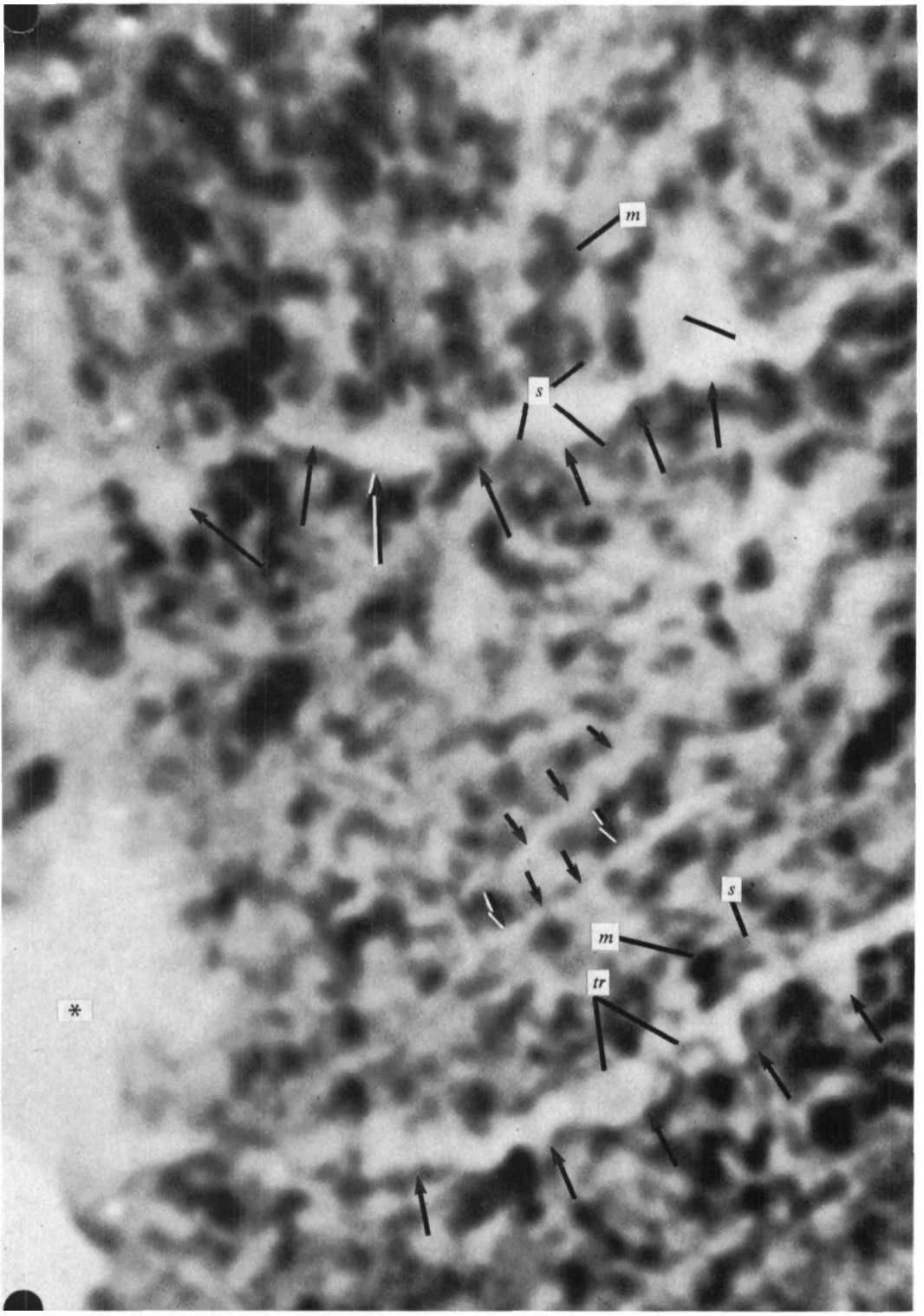


Fig. 3. Scanning transmission image of an unfixed unstained cryosection 1 μm thick, taken in the microanalyser at 50 kV. The section is partially hydrated. Rectum is cut tangentially with section passing through three of the four rectal papillae. All major tissue components can be seen. Contents of medulla have been largely lost during sectioning procedure. × 400.

Fig. 4. Scanning transmission image of an unfixed unstained cryosection 1 μm thick, taken in the microanalyser at 50 kV. The section is partially hydrated. Asterisk indicates a conical thickening of the basal lamina and arrows delineate narrow intercellular spaces. Some of these spaces contain profiles of tracheal branches (*tr*). Complex membrane stacks (*s*) and adjacent dense mitochondria (*m*) can be resolved. Water-deprived animal; × 8000.


m
s
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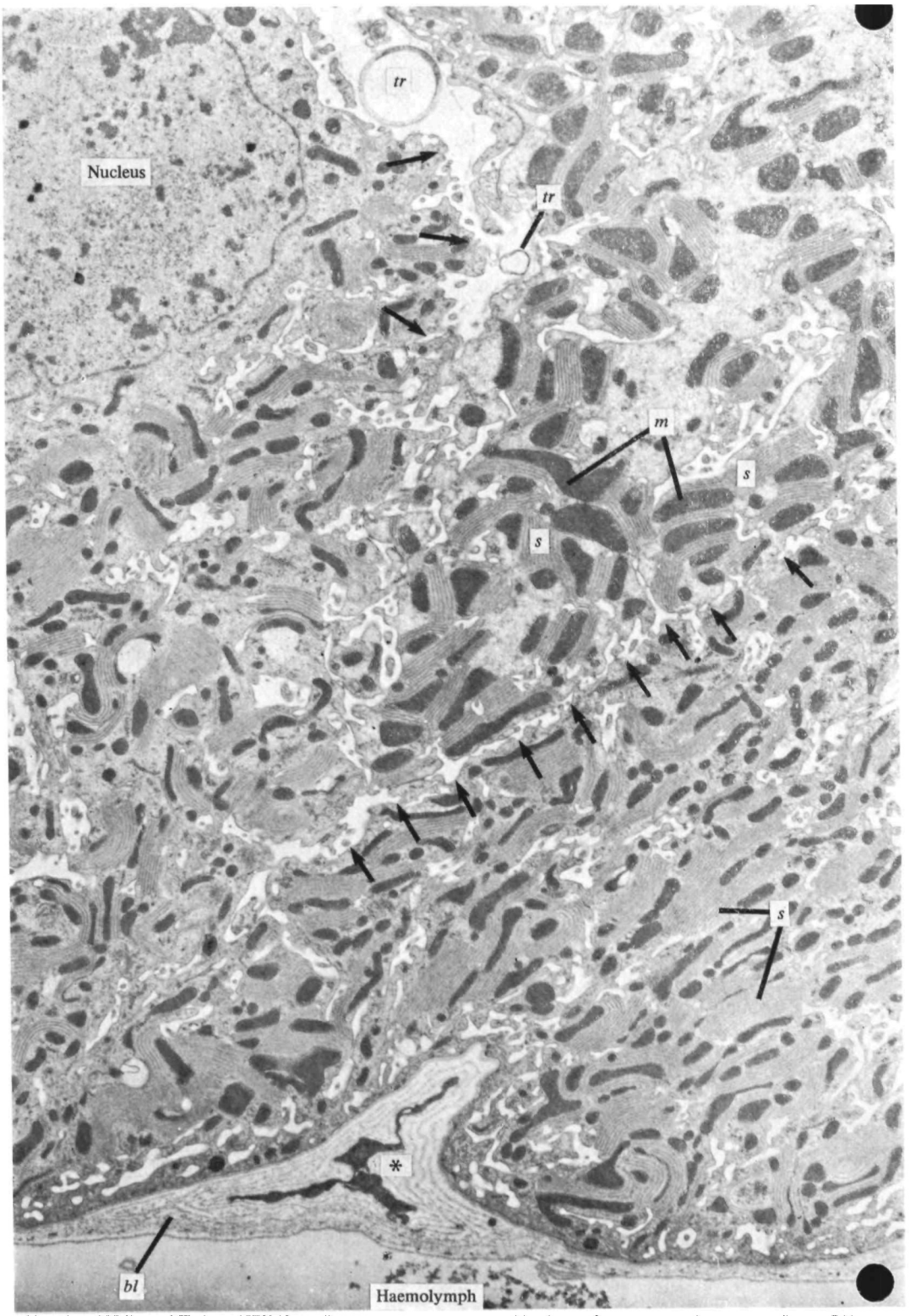
tr
Nucleus
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*
bl
Haemolymph

crystal formation during freezing and to improve sectioning properties at low temperatures. The copper pin with attached tissue was then quickly thrown into Freon-13 (monochlorotrifluoromethane) slush cooled to −180 °C by liquid nitrogen. The specimen was then transferred with pre-cooled forceps to a beaker of liquid nitrogen and placed in the chamber of a pre-cooled LKB CryoKit. The CryoKit attachment had been modified to provide better control of the chamber temperature (N. Cooper & B. L. Gupta, unpublished; Doty, Lee & Banfield, 1974; Kirk & Dobbs, 1976). The specimen was then cut into 1 μm thick sections with freshly broken glass knives. The knife and specimen temperatures were adjusted between −80° and −120 °C to obtain optimal cutting conditions. Sections were picked up with a pre-cooled eyelash attached to the end of an applicator stick, placed on an aluminized nylon film stretched over the end of a Duralium collar, and flattened by applying a polished copper disc stored in the cryochamber. The specimen holders were transferred to a custom-built storage and transport Dewar cooled with liquid nitrogen (see Gupta *et al.* 1977*a*; Gupta & Hall, 1979; Gupta, 1979 for details).

Analyses were carried out with a JEOL JXA-50A scanning microanalyser, fitted with a cold stage and anticontamination plate (Taylor & Burgess, 1977), employing the dextran-Ringer in the sections as the standard for quantification of the X-ray data, as previously described (Gupta *et al.* 1978*a*, Gupta *et al.* 1977*b*; Hall, 1979*b*).

RESULTS

Morphology of frozen sections

Major tissue components could be seen in scanning transmission images of frozen-hydrated sections (Fig. 3). Further details could be observed in partially hydrated sections (Fig. 4) at a resolution similar to that of a light microscope image of a 1 μm thick, Araldite-embedded methylene-blue-stained section (cf. Fig. 3 of Gupta & Berridge, 1966*a*). The most readily discernible features were the cuticle, apical fold region, nucleus, lateral membrane stacks with associated mitochondria, intercellular spaces and infundibulum. Their appearance in a conventional transmission electron micrograph is shown in Fig. 5.

Analysis of frozen sections

Successful analysis (Tables 1–6) was made of six recta (I to VI), prepared as described in Materials and Methods.

Cytoplasm

In this report we have included only those measurements where we are reasonably confident of the location of the electron probe; hence some of the sets of analyses are

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Fig. 5. Conventional transmission electron microscope image of cortical cells of rectal papilla of normal flies on emergence. Araldite section 100 nm thick, fixed and stained. Asterisk indicates conical thickening of basal lamina (*bl*). Arrows delineate narrow intercellular spaces, some of which contain profiles of tracheal branches (*tr*). Complex membrane stacks (*s*) and adjacent dense mitochondria (*m*) are resolved. A nucleus (*Nu*) appears in the micrograph. × 12000.

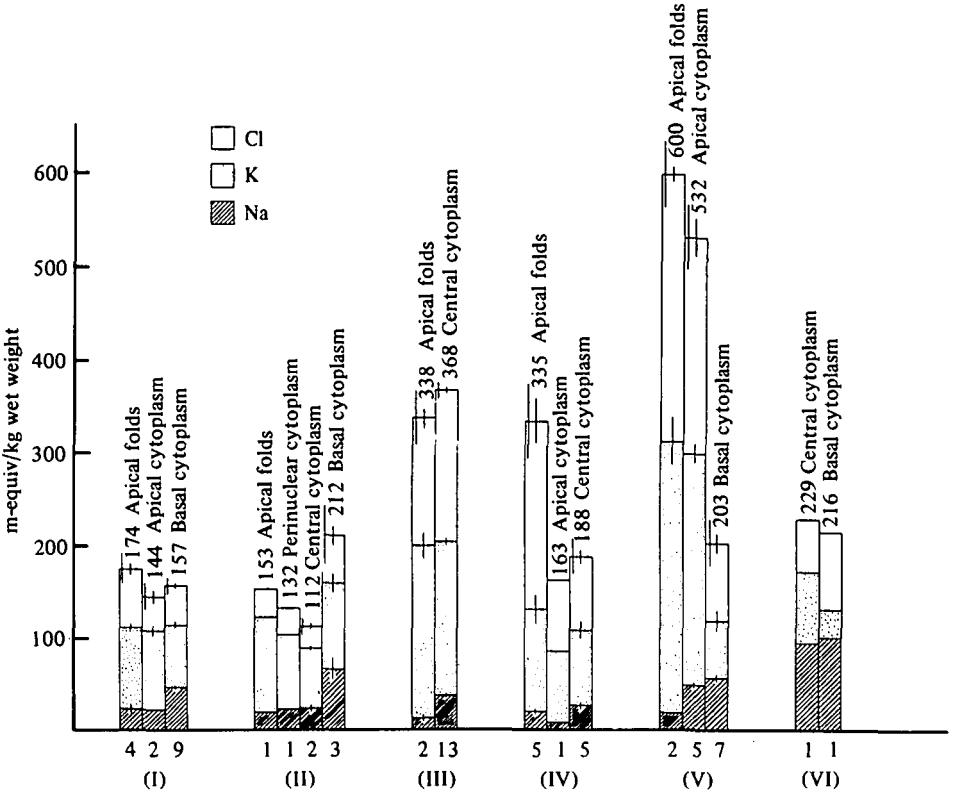

Fig. 6. Cytoplasmic concentrations in recta of six animals: (I) water-fed; (II) water-fed; (III) water-deprived; (IV) water-deprived, rectal lumen injected with 50 mM-NaCl, 100 mM KCl, 20% dextran; (V) water-deprived, rectal lumen injected with 100 mM KCl, 100 mM sucrose, 20% dextran; (VI) water-deprived, rectal lumen injected with 100 mM NaCl, 100 mM sucrose, 20% dextran. Numbers at top of columns are totals for Na + K + Cl concentrations. Numbers below columns are numbers of determinations. Averages ± 2 S.E. are shown for each element in centre of columns, while total electrolyte concentrations ± 2 S.E. are shown at top left of each column.

In most cases it has been possible to compare elemental concentrations in different parts of cells. In general, cytoplasm of water-deprived animals has a higher concentration of electrolytes than that of water-fed animals. Potassium concentrations are higher in apical fold regions, particularly in water-deprived animals. K content was higher than Na, except for animal VI which had been injected with NaCl.

more complete than the others. Furthermore, measurements of basal and apical cytoplasm and stacks were not always made from the same cell.

Fig. 6 summarizes the analyses of the cytoplasm in the different recta (see also Tables 1–6). In the water-fed animals (I and II) there is relatively little difference in the Na, K and Cl content of different parts of the cytoplasm, except that the Na concentration appears to be higher in the basal portions of the cells, nearest the infundibular space, particularly in II. The average K/Na ratios are shown in Table 7. Cytoplasmic K/Na ratios in recta from water-fed flies averaged about 2:1, while they were closer to 5:1 in water-deprived animals. Injection of an NaCl solution into the rectal lumen (rectum VI) was followed by a large influx of Na into the cells, and the K/Na ratio dropped to 0·6 (see also Gupta *et al.* 1978).

Table 1. *Water-fed animal I. Electrolyte concentrations*

	m-equiv kg^{-1} wet weight (means ± standard error)			
	Na	K	Cl	(*n*)
Lumen	13 ± 3	37 ± 8	38 ± 7	7
Cuticle	nd	16 ± 7	49 ± 7	3
Subcuticular space	13 ± 6	37 ± 8	44 ± 5	2
Apical folds	24 ± 5	88 ± 5	62 ± 7	4
Nucleus	24 ± 3	84 ± 7	29 ± 3	6
Apical cytoplasm	23 ± 1	85 ± 6	38 ± 10	2
Basal cytoplasm	47 ± 2	67 ± 4	43 ± 3	9
Apical stacks	27 ± 5	116 ± 6	81 ± 5	4
*(Extracellular channel of stacks)	(31)	(147)	(150)	
Basal stacks	73 ± 8	85 ± 7	133 ± 10	6
*(Extracellular channel of stacks)	(103)	(100)	(224)	
Intercellular spaces	64 ± 9	84 ± 11	142 ± 11	9
Intercellular sinuses	58 ± 10	89 ± 12	144 ± 24	6
Infundibulum near apex of papilla	65	47	140	1
Infundibulum near opening to haemolymph	72	53	114	1
Muscle	10	106	30	1

* Estimated value, assuming that 50% of the measured volume is contributed by the cytoplasm.

Table 2. *Water-fed animal II. Electrolyte concentrations*

	m-equiv kg^{-1} wet weight (means ± standard error)			
	Na	K	Cl	(*n*)
Lumen	20	95	142	1
Cuticle	5	12	60	1
Apical folds	20	103	30	1
Nucleus (apical)	19	94	20	1
Cytoplasm (perinuclear)	23	81	28	1
Cytoplasm	24 ± 4	65 ± 2	23 ± 4	2
Cytoplasm near infundibulum	67 ± 12	93 ± 11	52 ± 11	3
Stacks	93 ± 1	116 ± 15	104 ± 12	2
*(Extracellular channel in stacks)	(119)	(139)	(185)	
Mitochondrion	31	117	26	1
Intercellular space	91	90	143	1
Infundibulum near apex of papilla	147 ± 8	134 ± 24	171 ± 34	2

* Estimated value, assuming that 50% of the measured volume is contributed by cytoplasm.

In animals IV and V, in which the rectal lumen was injected with solutions containing 100 mM KCl prior to freezing, the K and Cl concentrations are extremely high in the apical fold region and also in the apical portion of the cytoplasm (Fig. 2).

Comparison of cytoplasm and intercellular spaces

For this comparison the X-ray data from the apical cytoplasmic folds have not been included because these folds are too far away from the membranes bordering the lateral intercellular spaces to affect any local osmotic gradients. In the water-deprived animals these apical cytoplasmic regions also had a high K and Cl content. If the data from these fields were to be included in this comparison, the conclusion below would not be affected (see *Appendix*).

Location of the probe in intercellular spaces was easier in water-deprived animals,

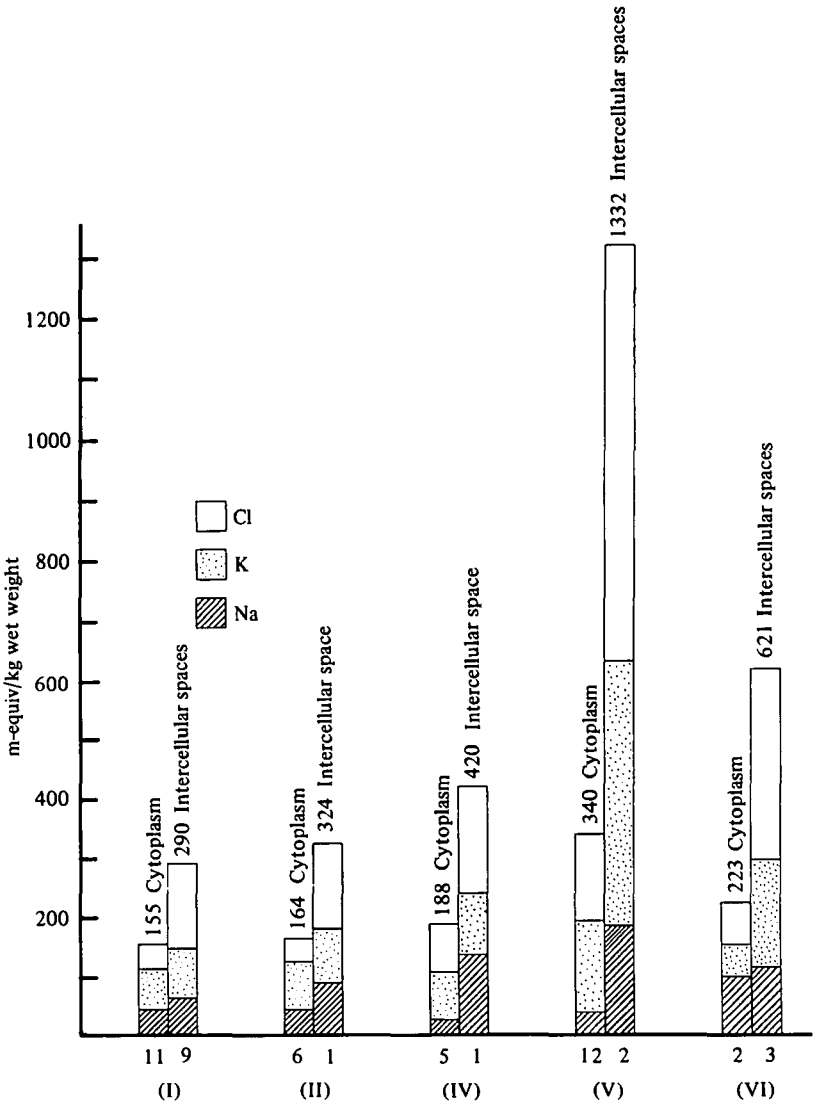

Fig. 7. Comparison of Na, K and Cl in cytoplasm and intercellular spaces, displayed as in Fig. 6. Na and Cl concentrations are always higher in intercellular spaces than cytoplasm, as are the totals of Na + K + Cl. Animal V, which was water-deprived and injected with KCl, has highest concentration in intercellular spaces.

where the spaces are larger (Berridge & Gupta, 1967; Wall *et al.* 1970). However, the contents of these larger spaces were frequently lost, apparently because they contain little organic matrix to bind them to the surrounding tissue.

The sum of Na + K + Cl concentrations was higher in the intercellular spaces than in the cytoplasm (Fig. 7). For example, in water-fed animal I the values are 290 ± 31 m-equiv kg^{-1} wet weight (range 240–357) and 155 ± 11 m-equiv kg^{-1} (range 130–176), respectively. The average individual contributions to the difference were 100 m-equiv Cl, 21 m-equiv Na and 14 m-equiv K. In recta II and IV, Na contributed more th

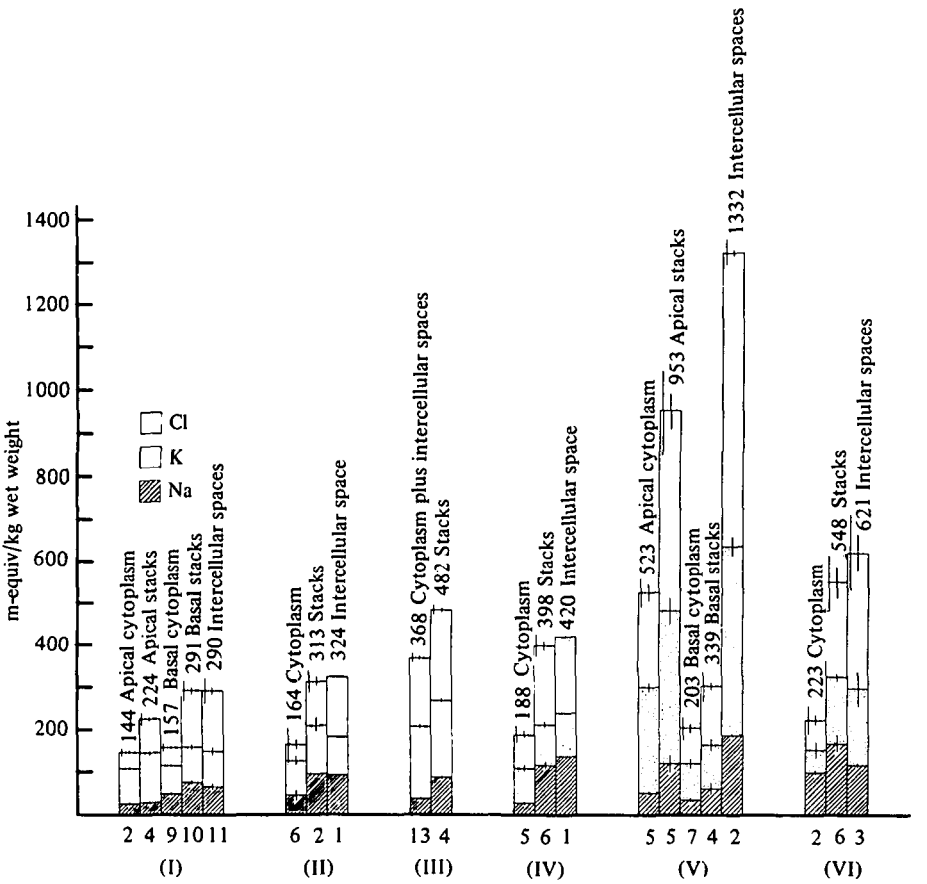

Fig. 8. Comparison of Na, K and Cl concentrations in membrane stacks, cytoplasm and intercellular spaces displayed as in Fig. 6. Total concentration in stacks appears to be higher than in cytoplasm and equal to or lower than that of intercellular spaces.

K to the difference between intercellular spaces and cytoplasm. In rectum V, which was injected with a KCl solution, the difference in K (450 m-equiv) exceeds that in Na (146 m-equiv). In rectum VI, which was injected with NaCl, there was also a larger difference in K (127 m-equiv) compared with Na (17 m-equiv).

Stacks

The image details in frozen sections are not generally adequate to resolve the individual components of the stacks (Fig. 4). However, by considering the stacks as separate units, it was found that the average values of $Na+K+Cl$ in the stacks are higher than those in the cytoplasm and lower than those in the intercellular spaces (Fig. 8). From this it appears that some component of the stacks accumulates electrolytes at a higher concentration than the cytoplasm. The most likely site of this accumulation is the extracellular channels. If it is assumed that the measured microvolumes from the stacks include an average of 50% cytoplasm and 50% extracellular space (see Fig. 2) then it is possible to apply simultaneous equations given by Gupta

Table 3. *Water-deprived animal III. Electrolyte concentrations*

	m-equiv kg^{-1} wet weight (means ± standard error)			
	Na	K	Cl	(*n*)
Lumen	30±3	107±6	382±29	3
Cuticle	20±3	73±5	262±26	4
Apical folds	14±3	187±10	137±12	2
Cytoplasm + spaces	38±2	167±4	163±7	13
Stacks	86±5	181±8	215±19	4
Infundibulum near apex of papilla	84±5	151±7	292±27	4

et al. (1978*a*) and estimate the concentrations of Na, K and Cl in the extracellular channels within the stacks. These estimates (in parentheses in Tables 1, 2, 4, 5 and 6) suggest that the values of Na + K + Cl in the extracellular channels in the stacks may be even higher than in the intercellular spaces. Unlike the average values in the stacks, the ratio of Na + K : Cl is nearly 1 in these estimated extracellular values, suggesting that the assumptions are correct.

The most complete sets of data are for recta I and V (Fig. 8), and in both cases it s possible to compare the cytoplasm and stacks in the apical and basal portions of the cells. In the water-fed animal (I) the stacks in the basal portion of the cells had a higher Na + K + Cl than those in the apical region. In the KCl-injected animal (V), the stacks in the apical region had more than 3 times the Na + K + Cl than the basal stacks. From Fig. 8 it can be seen that the high K and Cl content of the apical stacks may be related to the high KCl concentration of the apical cytoplasm.

The above comparison of microprobe data in total electrolyte content of Na + K + Cl in m-equiv kg^{-1} wet weight does not permit a complete assessment of osmotic gradients between various tissue compartments. The X-ray signals from various electrolyte elements are virtually independent of their state of osmotic activity and the continuum X-ray (see Gupta *et al.* 1977*a*) indicates total mass (i.e. residual dry mass + H_2O) in the microvolume under the electron probe.

The osmotic concentration of a fluid is determined by the total number of dissolved solute particles per unit volume of water and is given as m-osmoles kg^{-1} H_2O. To estimate the osmotic concentration in tissue compartments, and hence the osmotic gradients between them, we have converted microprobe readings of m-equiv kg^{-1} wet weight into m-osmoles kg^{-1} H_2O, by assuming (*a*) that all the water lost on drying the section is free water, (*b*) that all the measured elements are in free solution (see Oschman, 1978), (*c*) that fluids are electrically neutral (i.e. Na + K = anion) and (*d*) that no other solutes are osmotically significant (the X-ray energy spectra indicated that no other electrolyte elements were significant). The mass fractions in m-equiv kg^{-1} wet weight are then converted to m-equiv kg^{-1} H_2O by multiplication by the factor $1/1-f$, where f is the local dry weight fraction, which had values given in Table 8. Such conversion of microprobe data for *Calliphora* salivary glands has provided values which compared well with the parallel measurements using ion-selective microelectrodes (Gupta *et al.* 1978). The values for such calculated osmolalities of the fluids for recta I and V are given in Table VIII and follow a descending order in the route that the absorbed fluid follows (Berridge & Gupta, 1967; Wall, 1971; Gupta, 1976; Phillips, 1977). This table also includes K/Na ratios, which are not subject t

Table 4. *Water-deprived animal IV, lumen injected with* 50 *mM NaCl,* 100 *mM KCl,* 20% *dextran. Electrolyte concentrations*

	m-equiv kg^{-1} wet weight (means ± standard error)			
	Na	K	Cl	(*n*)
Lumen, injected solution	50	100	150	
Lumen, measured	35 ± 2	98 ± 4	146 ± 3	6
Epicuticle	14 ± 3	35 ± 5	185 ± 10	3
Endocuticle	10 ± 2	27 ± 4	165 ± 14	6
Subcuticular space	12 ± 1	21 ± 4	118 ± 10	8
Apical folds	22 ± 4	110 ± 13	203 ± 24	5
Apical cytoplasm	9	78	76	1
Cytoplasm	28 ± 4	81 ± 9	79 ± 8	5
Stacks	116 ± 10	95 ± 7	187 ± 10	6
*(Extracellular channel of stacks	(204)	(109)	(295)	
Intercellular spaces	138	102	180	1
Intercellular sinuses	64 ± 1	37 ± 12	106 ± 22	2
Bathing Ringer, measured	145	22	160	1
Bathing Ringer, known	132	20	158	
Muscle	16	113	15	1

* Estimated value, assuming that 50% of the measured volume is contributed by the cytoplasm.

many of the uncertainties in the quantification procedures (Hall, 1979*b*). The values for the Ringer bathing medium and rectal muscle are included for comparison.

Calculated osmolalites for the cytoplasm, intercellular spaces and intercellular sinus of rectum I are nearly equal to each other (Table 8). The fluid in the extracellular channels in the stacks has the maximum calculated osmotic concentration and appears to be 80 m-osmole kg^{-1} H_2O more concentrated than cytoplasm or intercellular spaces. Na + K + Cl of the absorbate (infundibular space near the opening into the haemolymph) is about 50 m-osmole kg^{-1} H_2O less concentrated than the cytoplasm, intercellular spaces, sinuses, and bathing Ringer, while about 2·5 times more concentrated than the lumen contents. However, as noted by Phillips (1964*b*, 1969, 1977) in *Schistocerca* and *Calliphora* and by Wall & Oschman (1970) in *Periplaneta*, the insect rectum also absorbs organic solutes such as amino acids and sugars, which constitute an important osmotic component of the absorbate in water-fed animals.

Thus in water-fed blowflies the hyperosmotic fluid generated in the stacks equilibrates with the surrounding tissue during passage through the larger intercellular spaces and sinus. However, this osmotic equilibration does not involve only the movement of water into the spaces. K/Na ratios of the absorbed fluid continuously decrease as it flows from the extracellular channels of the stacks through the intercellular spaces and infundibular spaces. Potassium, therefore, appears to return to the cells.

A similar pattern is seen for water-deprived animal V, where the rectum was injected with 100 mM KCl + 100 mM sucrose (Table 8). Since the rectum already contained concentrated excreta of mostly organic matter (Phillips, 1964*a*, *b*; 1969), neither the injected fluid nor the measured values give an indication of the real osmotic concentration of the lumen content. From previous work of Phillips (1969) on *Calliphora* and Wall & Oschman (1970) on the cockroach, the concentration of the rectal lumen contents from animals deprived of water is expected to exceed 500 m-osmole kg^{-1}

Table 5. *Water-deprived animal V, lumen injected with* 100 *mM KCl,* 100 *mM sucrose,* 20 % *dextran. Electrolyte concentrations*

	m-equiv kg^{-1} wet weight (means ± standard error)			
Hydrated section	Na	K	Cl	(*n*)
Lumen, injected solution	—	100	100	
Lumen, measured	30 ± 2	83 ± 8	120 ± 18	5
Endocuticle	27	128	210	1
Basal cytoplasm	20 ± 4	100 ± 11	82 ±	3
Stacks	63	177	175	1
Infundibulum, near papilla apex	117 ± 18	94 ± 4	247 ± 33	3
Bathing Ringer, measured	136 ± 26	40 ± 9	160 ± 3	3
Bathing Ringer, known	132	20	158	
Partly dehydrated section				
Lumen	45 ± 5	83 ± 13	147 ± 13	5
Cuticle	27 ± 1	77 ± 13	317 ± 26	4
Subcuticular space	19 ± 2	62 ± 14	203 ± 3	3
Apical folds	21 ± 3	292 ± 28	187 ± 8	2
Basal cytoplasm	33 ± 4	87 ± 12	83 ± 11	7
Apical cytoplasm	51 ± 3	249 ± 11	232 ± 21	5
Nucleus	8	110	44	1
Basal stacks	61 ± 13	103 ± 14	138 ± 8	4
*(Extracellular channel of stacks)	(89)	(123)	(193)	
Apical stacks	120 ± 21	360 ± 32	473 ± 43	5
*(Extracellular channel of stacks)	(207)	(653)	(863)	
Apical intercellular spaces	186 ± 21	450 ± 38	690 ± 53	2
Apical intercellular sinus	204	348	571	1
Infundibulum opening to haemolymph	87	51	148	2

* Estimated values, assuming that 50 % of the measured volume is contributed by cytoplasm.

H_2O. In electrolyte content the cytoplasm from the apical part of the cell (near the rectal cuticle) seems 550 m-osmole kg^{-1} H_2O more concentrated than the fluid in the lumen. However, this calculated osmolality of the apical cytoplasm is intermediate between those of the lumen and the extracellular channels in the stacks, which is 1720 m-osmole kg^{-1} H_2O. The concentrated fluid in the extracellular channels of the stacks is diluted to 345 m-osmole kg^{-1} H_2O by the time the fluid has flowed through the intercellular spaces and sinuses and reaches the infundibular space near the opening into the haemolymph. Again, K appears to be returned to the cell. Thus, in spite of the K/Na ratio in the extracellular channels of the stacks being twice as high as the ratio for water-fed animals, the K/Na ratio for the final absorbate for both animals I and V is similar. The values for rectum V given in Table 8 are derived from the apical part of the cells. For water-deprived animal V, the cytoplasm as well as stacks in the basal part of the cells and the infundibular space near the opening to the haemolymph had ionic concentrations similar to those in water-fed animals (see Tables 1 and 5).

Although the number of observations for recta I and V are limited, an estimate can also be made of changes in Na and Cl concentration of the fluid during its passage from the stacks to the haemolymph, and a better estimate can be made of K concentration (Table 9). In rectum I and calculated osmolality of the emergent fluid near the infundibular opening is 66 % that of the fluid in the stacks, and in rectum V it is 20 %. If this change were only by dilution with H_2O, one would expect the same percentage

Table 6. *Water-deprived animal VI, lumen injected with* 100 *m*M *NaCl,* 100 *m*M *sucrose,* 20% *dextran. Electrolyte concentrations*

	m-equiv kg^{-1} wet weight (means ± standard error)			
	Na	K	Cl	(*n*)
Lumen, injected solution	100	—	100	
Lumen, measured	111 ± 13	30 ± 7	130 ± 17	6
Epicuticle	62	12	86	1
Endocuticle	118	36	98	1
Cytoplasm + spaces	120 ± 9	76 ± 6	72 ± 12	3
Cytoplasm	99 ± 3	53 ± 16	71 ± 10	2
Stacks	169 ± 21	154 ±	225 ± 38	6
*(Extracellular channel of stacks)	(242)	(232)	(393)	
Intercellular sinuses	117 ± 5	180 ± 42	324 ± 45	3
Muscle	2 ± 1	120 ± 2	23 ± 4	5

* Estimated values, assuming that 50% of the measured volume is contributed by cytoplasm.

change in the individual values for Na, K and Cl. However, Table 9 shows that in both animals the concentration of Na is about 60% higher than the expected value. Chloride is about 10% less, but K is about 50% less than the expected values. The estimates show that the absorbed fluid, during its passage in the intercellular spaces and sinuses, gains not only H_2O but also Na. At the same time the absorbed fluid loses K and perhaps some Cl.

DISCUSSION

It was proposed by Gupta & Berridge (1966*a*) and Berridge & Gupta (1967, 1968) that the apparent active transport of water in the rectal papillae of *Calliphora* (Phillips, 1964*a*, 1965) can be explained by some form of local osmosis (Curran, 1960). The model of local osmosis proposed by Gupta & Berridge (see Introduction) predicted that in recta of insects such as blowfly, cockroach and locust (Wall & Oschman, 1975), the fluid in the intercellular spaces and sinuses within the epithelium of rectal papillae and pads would be more concentrated than the cytoplasm and rectal lumen. So far, the study by Wall *et al.* (1970) on the analyses of micropuncture samples from the intercellular sinuses in the cockroach rectal pads has remained the only direct evidence to support the predictions of local osmosis. Hill (1975) has questioned the results obtained during micropuncture sampling because of the danger of tissue damage and mixing of aliquots from narrow spaces. Since water absorption in the cockroach, locust and blowfly can occur from hyperosmotic solutions of impermeant sugars injected into the rectal lumen, the finding of Wall *et al.* (1970) is consistent with the idea that fluid absorption is by solute–solvent coupling (Phillips, 1977) but does not afford proof.

Goh & Phillips (1978) have recently shown that *in vitro* preparations of everted recta from the locust, *Schistocerca gregaria*, can only sustain fluid absorption from an impermeant sugar solution if a permeant monovalent ion (Na, K, Cl) is present in the lumen. Furthermore, as observed previously, the final absorbate always contained substantial amounts of ions even when the fluid added to the lumen side was a pure sugar solution. The results of the present study provide compelling support for the local osmosis model and add further details to the mechanism of fluid absorption in *Calliphora* rectum.

Intercellular fluid

If electrolytes are the primary basis of hypertonicity in the intercellular spaces, then the local osmosis hypothesis predicts that their average concentration in the intercellular spaces will be higher than in other tissue compartments. In all six recta examined here this appears to be the case (Fig. 7). Sodium plus potassium concentrations of these large intercellular spaces more or less equal their chloride concentration (Tables 1–6). The dry-mass fraction in most of these spaces was nearly zero. The osmolality of the fluid in these spaces should therefore be nearly equal to their Na + K + Cl content plus some contribution from non-electrolyte solutes, as found by Wall & Oschman (1970) in the cockroach. Except in water-deprived animal V, where a KCl solution had been injected into the rectal lumen before freezing, both NaCl and KCl contribute more or less equally to the osmolalities of the fluid in the intercellular spaces.

As explained in Results, a more realistic estimate of osmolality of fluids is provided for Animals I and V in Table 8. The calculated osmolality, 2(Na + K), of the various samples can be compared with the results obtained with the cockroach rectum by Wall & Oschman (1970). Similarity in Na + K + anion values in various fluids between the blowfly and cockroach is found in both water-fed and water-deprived animals. For example, the contribution of Na + K + anion for the fluid in the infundibular space near the papillar tip is 280 m-osmole kg^{-1} H_2O for the water-fed blowfly (see Table 8) which is close to that (*ca.* 300 m-osmole) in the comparable fluid (anterior sinus) of the rectum of water-fed cockroaches with a measured osmolality of 363 m-osmole kg^{-1} H_2O (Wall & Oschman, 1970). In the cockroach, Wall (1977) found that the Na:K ratio of intercellular fluid changes according to the composition of the fluid injected into the rectum of water-deprived insects. Similar results are obtained in the present study (see Tables 4, 5 and 6). There is good agreement between the results from the present microprobe study of *Calliphora* rectum and the previous estimates by osmometry and flame photometry of micropuncture samples from the cockroach rectum. A close similarity of results from these completely different species of insects is not surprising. In both *Calliphora* and *Periplaneta* the haemolymph composition is similar. In both species fluid absorption under conditions of water deprivation can concentrate rectal contents to more than 1000 m-osmole (Phillips, 1969; Wall & Oschman, 1970).

Studies of cockroach rectum by Wall *et al.* (1970) do not provide information either on the primary sites of solute transport or on the actual concentrations within the narrow intercellular channels of the rectal pads. In the present study using the microprobe, we have found that in all six samples the average concentration of Na + K + Cl in the stacks (Fig. 8) is higher than that in the cytoplasm. As explained in the Results, these measurements of the stacks must include a variable (according to the size and position of the 200 nm electron probe) contribution from the cytoplasm. This is reflected in Cl values being generally lower than Na + K. The estimated concentrations of Na, K and Cl in the extracellular channel of the stacks, given in parentheses in Tables 1, 2, 4, 5 and 6, show that the highest concentrations of all three electrolytes are present in these channels. This is entirely consistent with the localization of major solute pumps on these membranes as demonstrated by a histochemical localization of Mg-ATPase (Berridge & Gupta, 1968). In freeze-fracture replicas (B. L.

Table 7. *Average K/Na ratios in cytoplasm*

Animal	Condition	Injection	Average K/Na
I	Water-fed	—	2·6
II	Water-fed	—	2·0
III	Water-deprived	—	4·4
IV	Water-deprived	Na + K	5·8
V	Water-deprived	K + sucrose	5·0
VI	Water-deprived	Na + sucrose	0·6

Gupta & S. K. Malhotra, unpublished observations) these membrane stacks together with the apical membrane folds also contain the highest number of intramembranous particles per unit area of the surface (see also Lane, 1979; Flower & Walker, 1979).

Berridge & Gupta (1967, 1968) speculated that K must be the major ion secreted in the stacks because (*a*) the fluid secreted by *Calliphora* Malpighian tubules which enters the rectal lumen is mainly KCl (see Berridge, 1968; Gupta, 1976) and (*b*) K is reabsorbed from the rectum of *Schistocerca* ten times faster than sodium (Phillips, 1964*b*). However, it now looks as if these so-called potassium pumps in many insect tissues are less orthodox than the conventional Na–K exchange pumps (Glynn & Karlish, 1975). The cation pumps in insect tissue can transport K or Na or both, according to the activity of these ions in the adjacent cytoplasm (Maddrell, 1977). Thus the unstimulated Malpighian tubules of *Rhodnius* secrete a fluid which is mainly KCl; after stimulation by 5-hydroxytryptamine the secreted fluid contains Na and K in equimolar ratios and the average cytoplasmic concentration of Na goes up from 22 to 42 mM (Gupta *et al.* 1976). Therefore it is not surprising that in most cases the extracellular fluid in the stacks contains both Na and K. The actual ratio of these ions varies and probably reflects the contents of the lumen and the physiological state of the rectum. When KCl was injected into the lumen, the extracellular fluid in the stacks had 3·2 times more K than Na (Tables 5 and 8). However, when NaCl was injected into the rectal lumen, the fluid in the stacks contained Na and K in equimolar ratio. In animal V (KCl-injected rectum), the lumen contents contained Na (about 30 m-equiv) and in animal VI (NaCl-injected rectum), the lumen contents contained K (about 30 m-equiv), presumably from the pre-existing faecal contents.

If electrolytes transported by the lateral membrane stacks are primarily responsible for drawing water by local osmosis, the concentrations of fluid at these sites must exceed the osmotic concentration of the rectal lumen. This appears to be generally the case. Maximum electrolyte concentrations were found in the apical stacks of water-deprived animal V where KCl had been injected into the lumen (Tables 5 and 8). The measured concentration of electrolytes in the lumen was 250 m-equiv kg^{-1} wt weight (Table 5), which only reflects the composition of the injected fluid. Since the animals had been deprived of water, the actual osmotic concentration of the lumen contents may have been very high due to organic excreta. Some estimate of the lumen contents in water-deprived animals can be made from animal III. Here the lumen contained 382 m-equiv kg^{-1} wet weight of Cl. This can be compared with the maximum Cl value of 394 m-equiv kg^{-1} H_2O measured by Phillips (1969) in water-deprived *Calliphora*. Even if neutral organic solutes and dry mass are ignored, the Cl + cation may represent about 764 m-osmole kg^{-1} H_2O as the minimum osmolality of the rectal contents in water-deprived flies prior to injection. An osmolality of nearly 1000 m-

Table 8. *Comparison of calculated osmolality of Na+K+anion and K/Na ratios for water-fed animal I and water-deprived animal V, lumen injected with* 100 mM *KCl,* 100 mM *sucrose,* 20% *dextran*

	Animal I		Animal V	
	(Na+K)×2 (m-osmole kg^{-1} H_2O)	K/Na	(Na+K)×2 (m-osmole kg^{-1} H_2O)	K/Na
Lumen	100	2·8	250[a]	2·8
Cytoplasm[b]	296	2·2	800	4·9
Extracellular channels in stacks	381	1·8	1720[d]	3·2
Intercellular space[c]	300	1·3	1272[d]	2·4
Intercellular sinus	294	1·5	1104[d]	1·7
Infundibular space near papillar tip	280	0·7	527[e]	0·6
Infundibular space near opening into haemolymph	250	0·7	345[e]	0·6
Bathing Ringer	320	0·15	320[f]	0·15
Muscle, rectal[b]	309	10·6	352[g]	7·2

[a] Real osmolality is expected to be at least 700 m-osmoles (see p. 35).
[b] Residual dry mass about 25% of wet weight.
[c] Residual dry mass about 8% of wet weight.
[d] Apical part of cells.
[e] Residual dry mass about 20% of wet weight.
[f] Contained 20% w/v dextran.
[g] Data from water-deprived animal IV.

osmole has been recorded in the rectal lumen in water-deprived flies. Therefore an electrolyte concentration of 1720 m-equiv kg^{-1} H_2O at the primary sites of transport, the stacks, is not surprising. In *Tenebrio*, the cryptonephric Malpighian tubules can secrete a solution containing nearly 2 M KCl into the lumen (Ramsay, 1964; Grimstone, Mullinger & Ramsay, 1968).

In contrast, the average concentration of fluid in the stacks of water-fed animal I (381 m-osmole kg^{-1} H_2O) is much lower than that of water-deprived animals. In both water-fed cockroaches (Wall & Oschman, 1970) and water-fed blowflies (Phillips, 1969), a watery excrement is formed that is considerably hypo-osmotic to the haemolymph. In water-fed cockroaches the rectum absorbs ions but seems to be relatively impermeable to water (Wall, 1971; Wall & Oschman, 1970). In the present study, the lumen contents in animal I had a Cl value of 38 m-equiv kg^{-1} wet weight. This value is similar to a maximum Cl value of 28 m-equiv kg^{-1} H_2O in water-fed *Calliphora* measured by Phillips (1969). The osmolality of the rectal contents measured by Phillips was about 70 m-osmole compared to 100 m-osmole estimated here (Table 8).

The value of 381 m-osmole for the osmolality of the extracellular fluid in the stacks of water-fed animals is 80 m-osmole higher than the estimated values for the cytoplasm or large intercellular spaces and sinuses. Our microprobe measurements of other epithelia which transport isotonic fluid (*Calliphora* salivary glands, Gupta *et al.* 1978*a*; rabbit ileum, Gupta, Hall & Naftalin, 1978*b*) also demonstrate a similar magnitude of hypertonicity in the relevant sites of solute–solvent coupling (for further discussion see Gupta & Hall, 1979).

Table 8 shows that in both recta under two extreme conditions of fluid transport, the primary fluid produced in the stacks becomes less concentrated as it moves down the complex labyrinth of intercellular spaces.

Recycling of solutes

The shape of *Calliphora* rectal papillae in the form of a hollow cone stuffed with medullary tissues means that most of the radially arranged cortical cells have no direct access to the haemolymph (Gupta & Berridge, 1966*a*). The basal cell membrane in cortical cells constitutes only about 2% of the total cell surface and in most cases faces the infundibular space (Berridge & Gupta, 1967). It has been argued that to maintain their normal ionic content, especially high K, cortical cells must reabsorb K from the absorbate as the fluid moves from the stacks to the infundibular opening (Berridge & Gupta, 1967). Table 9 shows that in water-fed animal I the fluid from the stacks loses about 30 m-equiv of K (about 40%) but gains the same amount of Na before emerging into the haemolymph, so that there is no net reabsorption of ions by the cells. In water-deprived animal V, on the other hand, the cells reabsorb 80 m-equiv of K (about 60%) and again lose about 30 m-equiv of Na to the absorbed fluid. A small amount of Cl may also be reabsorbed. In animal V this amounts to net reabsorption of 100 mM of ions or about 25% of the total electrolytes of the primary fluid from the stacks. Studies on the cockroach rectum in situ (Wall *et al.* 1970; Wall & Oschman, 1970) and locust rectum *in vitro* (Phillips, 1977) using osmometry and flame photometry also suggest that ions are recycled from the absorbed fluid back into the tissue. Phillips (1977) has found that in locust rectum Na, K and Cl are all recycled from the absorbate back into the cells.

From the changes in K/Na ratios in Table 8 it seems that in both animals I and V the K–Na exchange is almost complete by the time the fluid reaches the infudibular space. In *Calliphora*, therefore, most of the reabsorption occurs along the large intercellular spaces and sinuses within the cortical epithelium. Berridge & Gupta (1968) did find in their histochemical preparations for Mg-ATPase that some deposits (although sparse) were formed on the lateral cell membranes forming the large intercellular spaces.

In addition to an Mg-ATPase, the rectal epithelium contains an ouabain-sensitive Na–K–ATPase (Tolman & Steele, 1976; Peacock, Bowler & Anstee, 1976). Peacock (1976) has also found that in locust rectum the activity of both these enzymes is stimulated by corpus cardiacum extracts. The hormonal control of rectal absorption in insects is now well established (Wall, 1967; Maddrell, 1971; Phillips, 1977). Therefore, it is likely that the K–Na exchange from the fluid in the large intercellular spaces may be carried out by a conventional Na–K–ATPase. As illustrated in Tables 8 and 9, the composition of the fluid emerging into the haemolymph is relatively constant, which suggests that the mechanism for K–Na exchange could be self regulatory. Sodium–potassium exchange ratios of the conventional 'Na pump' can also vary over a wide range according to the composition of the fluid bathing the two surfaces of the membranes (Glynn & Karlish, 1975).

However, K reabsorption cannot be the whole explanation of solute economy in blowfly recta. As in the cockroach (Wall, 1971), Na is the major cation in the final absorbate. Except in NaCl-injected recta, the general level of Na in the cytoplasm is low (around 30 m-equiv kg^{-1} wet weight, see Tables 1–5). As mentioned earlier, Goh & Phillips (1978) have also shown that in the locust rectum *in vitro* absorption of water from impermeant sugar solutions in the lumen stops after 1 h even when normal

Table 9. *Estimated changes in Na, K and Cl concentration of absorbate during passage from stacks to infundibular opening*

	Stacks[a]	Emergent fluid		
	Measured concentration (m-equiv kg^{-1} H_2O)	Expected[b] concentration (m-equiv kg^{-1} H_2O)	Mesured concentration (m-equiv kg^{-1} H_2O)	% Measured/ expected concentration
		Animal I		
Na	66	44	72	164
K	123	81	53	65
Cl	187	123	114	93
Total	376	248	239	96
		Animal V		
Na	207	54	87	161
K	653	131	51	39
Cl	863	177	148	84
Total	1713	372	286	77

[a] Values from Table 1 averages of numbers in parenthesis.

[b] For animal I, expected concentration values obtained by taking 66 % of measured concentration of stacks; calculated osmolality from Table 8 of emergent fluid (infundibular space near opening into haemolymph) is 66 % of that of fluid in extracellular channels of stacks.

For animal V, expected concentration values obtained by taking 20 % of measured concentration of stacks; calculated osmolality from Table 8 of emergent fluid is 20 % of that of fluid in extracellular channels of stacks.

Ringer is bathing the haemolymph side. Wall & Oschman (1970) postulated that Na could diffuse back into the cells from the absorbate as it flows along the tracheal indentations and the subepithelial (or sub-muscular) sinus. Back-diffusion of solutes from the haemolymph into the lumen may also be important (Phillips, 1964*b*, 1965, 1977; Goh & Phillips, 1978). That the ionic composition of the rectal lumen has a dominant effect on the composition of fluids in the cortical epithelium is shown in animals V and VI.

Cytoplasmic ions and route of water absorption

In early work it was assumed that the hyperosmotic interspaces of a local osmosis system were tightly sealed from the lumen by appropriate cell junctions (Tormey & Diamond, 1967; Diamond, 1979). Water flow into the interspace in response to the osmotic gradient created a slightly positive hydrostatic pressure and prevented fluid from entering through the open end of the interspace (Curran & Mackintosh, 1962; Diamond & Bossert, 1967). Most, if not all, of the water was then expected to enter the interspaces via the cytoplasm. In epithelia which transport isosmotic fluid (e.g. vertebrate gall bladder, small intestine, proximal kidney tubules, insect Malpighian tubules) the cytoplasm was nevertheless considered to be isosmotic with the fluid in the lumen and the blood. Therefore it was difficult to see how the cytoplasm could remove water preferentially from either the lumen or blood. However, it is now thought that in both vertebrate and insect epithelia which transport isotonic fluid, the

cell junctions are leaky (Staehelin, 1974; Lord & DiBona, 1976; Lane, 1979) and allow a considerable flow of ions and water through the paracellular route (e.g. Sackin & Boulpaep, 1975; Gupta & Hall, 1979). In epithelia which can maintain high osmotic gradients across them, the relevant cell junctions are thought to be tight, and most of the water and ions must therefore move through the cells. This would require some part of the cell to be hyperosmotic to the lumen, the luminal and lateral plasma membrane to be permeable to water, and the basal plasma membrane relatively impermeable to water in the case of an absorbing epithelium. Water would then flow through a cellular route and into the interspace in response to an osmotic gradient.

As discussed above, most of the cortical epithelial cells in *Calliphora* rectal papillae are not in direct contact with the haemolymph. The cortical cells at the base of the papilla are in contact with the haemolymph and possibly are isosmotic to the haemolymph. All the cortical cells of the papilla are in communication with each other via gap junctions (Lane, 1979). So, theoretically, it is possible for all the cells to be isosmotic with the haemolymph. However, the cell body of the large cortical cells is extensively carved up by lateral membrane folds, intercellular channels, spaces and sinuses, which must restrict diffusion and prevent rapid equilibration with the haemolymph. Berridge & Gupta (1967) thought that cell cytoplasm may also have osmotic gradients, so that the apical portions between the intercellular spaces and the lumen have an intermediate osmolality.

In the present study it was found that the cytoplasmic electrolyte concentrations vary in the basal and apical parts of the cells among the different animals (Figs. 6–9). Some of these variations are probably due to the difficulty of resolving true cytoplasm from other small structures (see previous sections). However, it is also likely that many of these differences are due to changes in cytoplasmic electrolyte content that occur during water absorption, as the excreta became concentrated and thus reflect the composition of the fluid surrounding the cells.

As noted above, the physiological evidence suggests that cortical cells mostly draw ions and water from the rectal lumen through the apical surface. The average electrolyte concentration in the apical membrane folds tends to be higher than either the subcuticular space or the apical cytoplasm. As in the stacks, the apical folds (Fig. 2) include both the cytoplasmic channels and extracellular channels (Berridge & Gupta, 1967), and the real electrolyte concentrations in the cytoplasmic channels may be much higher than these average values. These high electrolyte concentrations in the apical parts of the cytoplasm could provide an osmotic gradient that would draw water from the lumen into the cells. The 12 nm particles on the cytoplasmic surface of the apical folds (Gupta & Berridge, 1966*b*) and possibly a K–Mg stimulated ATPase (Berridge & Gupta, 1968) could be responsible for producing these high concentrations. Although the data are limited, at least in animals I and V (Table 8), it seems that the osmolality of the cytoplasm is intermediate between the lumen and intercellular spaces.

However, wherever measurements were possible, it was found that the circumnuclear cytoplasm and the nucleus had much lower electrolyte concentrations than elsewhere in the cells (e.g. Table 5), and these concentrations were similar to those measured by microprobe in other transporting epithelia (Gupta, 1976; Gupta *et al.* 1976, 1977*a*; Gupta *et al.* 1978*a*; Gupta Hall & Naftalin, 1978*b*; Rick *et al.* 1978*a*, *b*).

If all the measured variation in the cytoplasmic concentrations of electrolytes is real, it is not surprising that the average electrolyte concentration of rectal tissues as measured by flame photometric methods does not suggest the presence of any significantly high electrolyte gradients (Phillips, 1977; Stobbart, 1968; Wall, 1977) in the cells. It is not clear how such intricate gradients of electrolytes are maintained within the cell cytoplasm. It is widely (but not unanimously) held that the cytosol in all parts of the cell body is in diffusion equilibrium. However, microprobe measurements from other tissues have revealed variations in the ionic concentrations from one part to another within the cytoplasm (Bacaner *et al.* 1973; Gupta *et al.* 1978*b*; Gupta & Hall, 1979). Gradients of ionic activities and electrical potentials have also been measured by intracellular microelectrodes (Zeuthen, 1978). Clearly this problem requires further investigation.

Because the average concentrations of tissue electrolytes in cockroach and locust recta were not higher than the lumen or haemolymph, it has been proposed that non-electrolytes and/or the structuring of water to reduce intracellular water activity contribute to an increased osmolality in the cells (Wall, 1977; Phillips, 1977). Neither of these can be detected by microprobe. Alternatively, it has been proposed that water may move through the leaky cell junctions at the luminal side from the lumen directly into the hypertonic interspaces (Phillips, 1977; Lane, 1979).

In *Calliphora* it has been found (B. L. Gupta, unpublished) that when ligated recta of water-fed flies were incubated in solutions containing ionic lanthanum (2 mM $LaCl_3$ in Tris-Ringer) the marker infiltrated into septate junctions and reached some of the intercellular channels. However, in water-deprived flies the lanthanum tracer, which was largely concentrated in the thick basal lamina adjacent to the junctions, reached only a little way into the septate junctions and did not reach the intercellular spaces. Opposite results were obtained when the solution was injected into the rectal lumen. These results suggest that in water-fed flies the septate junctions on the basal surfaces are leaky (as also found by Lane, 1979), but in water-deprived flies apical junctions only may be leaky.

While permeability to lanthanum does not necessarily demonstrate the real permeability properties of cell junctions to monovalent ions and water *in vivo*, these results appear to fit the observations discussed above. Under diuretic conditions the insect needs to conserve ions lost by Malpighian tubules but not water. The apical junctions therefore need to be relatively impermeable in order to minimize movement of water from the lumen into the hypertonic interspaces and back-diffusion of ions from these spaces. Under these conditions permeable junctions on the basal surface will allow the cortical cells better access to the haemolymph and therefore better osmotic and ionic equilibration with it (see above). As noted above, all the cells in water-fed animals do seem to be more homogeneously isotonic with the haemolymph. The water entering from the haemolymph into interspaces may be essential to flush the ions being extruded by the stack membranes. Under diuretic conditions this arrangement will amount to a 'recycling' of tissue water and lead to a net absorption of ions from the lumen. The opposite will be true under conditions of water stress. In water-deprived animals it is further possible that the reabsorption functions may be concentrated in the apical part of the cortex and cells. The cells on the basal side may shut off partly by the changes in the membrane geometry, such as the collapse of narrow channels

noted in fasting flies (Berridge & Gupta, 1967; Wall & Oschman, 1975) and by uncoupling of gap junctions. This could also be an explanation of the differences between basal and apical regions in animals IV and V (Fig. 6). Like the permeability of coupling or communicating junctions (Loewenstein & Rose, 1978), the permeability of tight and septate junctions need not be patent but may modulate to meet the metabolic requirements of the tissue. There is evidence that ionic coupling between epithelial cells in insects may be locally modulated by hormones (Caveney, 1978). The trans-epithelial permeability of vertebrate tight junctions (DiBona & Civan, 1973) and invertebrate septate junctions (Lord & DiBona, 1976) can change according to the direction of trans-epithelial gradients. Bentzel & Hainau (1979) have shown that both structure and permeability of the 'leaky' tight junctions of *Necturus* gall bladder can be regulated by the cells.

Some technical aspects concerning the validity of electron microprobe X-ray analysis are further discussed in an Appendix to this paper.

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APPENDIX

Validity of results

The acceptance of the Na, K and Cl measurements by our microprobe technique depends on the validity of different critical steps in the method. We do not intend to discuss here the rationale of our technique, which has been described and discussed in many recent articles (e.g. Gupta, 1979; Hall, 1979*a*, *b*; Hall & Gupta, 1979). References and discussion can be found in two recent volumes (Lechene & Warner, 1979; and *Low Temperature Biological Microscopy and Microanalysis*, The Royal Microscopical Society, Oxford, 1978).

More specifically, the critical problem in the present study is the reliability of our comparisons of cytoplasmic and intercellular space composition. First, to what extent can solutes migrate between the cytoplasm and intercellular spaces during freezing and subsequent steps in preparing the samples ? In all of the tissues we have studied, extremely sharp transitions occur at the cell surface from the high K, low Na in the cytoplasm to the high Na, low K of the bathing medium. Similar results have been obtained by others who have studied muscle (Somlyo *et al.* 1979), red blood cells (Tormey, 1977; Jones *et al.* 1978), frog skin (Rick *et al.* 1978*a*) and toad urinary bladder (Rick *et al.* 1978*b*). It therefore appears that the steep concentration gradients across the cell surface are preserved by rapid freezing, and that the high electrolyte concentrations observed in intercellular spaces are not due to diffusion of solutes from the cytoplasm during freezing.

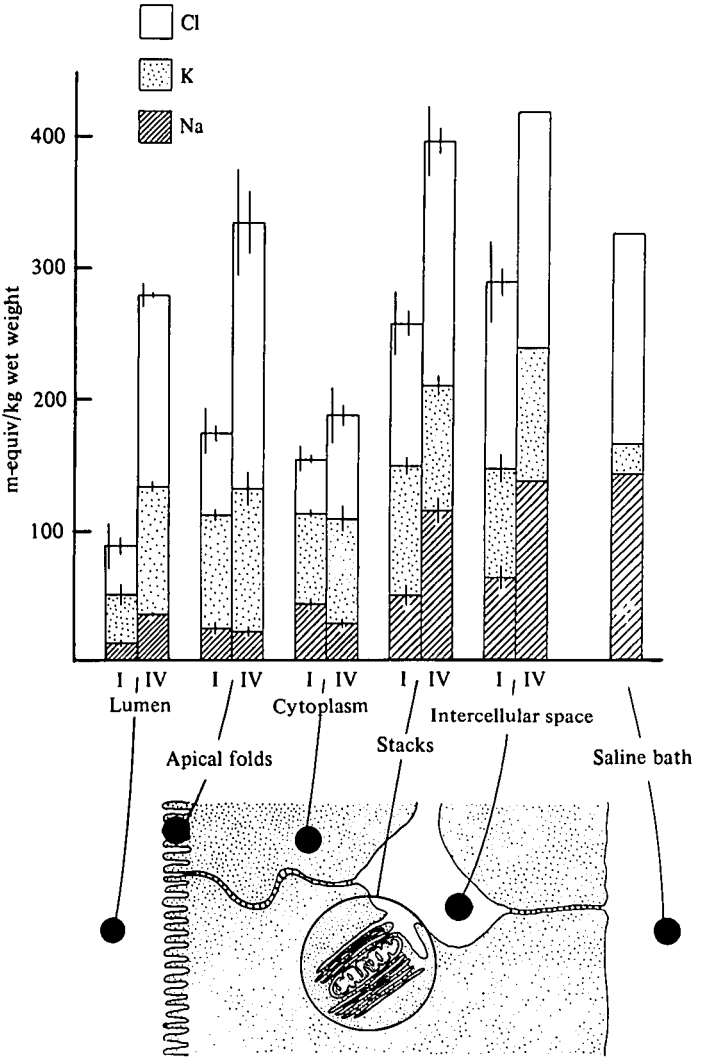

Fig. 9. Comparison of a water-fed (I) and a water-deprived (IV) animal displayed as in Fig. 6. The total of Na + K + Cl is higher in the water-deprived than in the water-fed animal.

A related problem is the possible migration of electrolytes along the intercellular channels. It is clear from Fig. 9 that intercellular fluid has a different composition (i.e. much higher K) than the bathing medium. The high total electrolyte content of intercellular fluid (e.g. in animals IV, V and VI) could be an artifact caused by migration of electrolytes from the infundibulum or from the bathing fluid, if such migration can occur against a concentration gradient and be preferential for K rather than for Na. There are two ways this might happen. First, it has been suggested that as the freezing front advances through the tissue, some of the solutes that are excluded from the ice crystal lattices may be mechanically swept ahead into the unfrozen phase (see Hall *et al.* 1974, pp. 204, 205), or may exert an osmotic pressure on adjacent regions, drawing water toward the crystallization sites (Rebhun & Sander, 1971). It has

been our experience that this type of solute dislocation can occur, but that it is always accompanied by severe ice crystal damage. When areas that are filled with large ice crystals are probed, very low signals are obtained for the various electrolytes, indicating that as the water was frozen solutes were pushed away into neighbouring areas. Severe ice crystal damage is readily detected in the imaging system, and specimens showing such damage are rejected. Moreover, if solutes were dislocated by the mechanism proposed above, this process would likely occur more readily in the rectal lumen, which is the last part of the tissue to freeze and which might therefore experience slower freezing. We find, however, that the measured concentrations of the electrolytes in the lumen agree closely with the known concentrations of the injection fluid. Moreover, the lateral membrane stacks, intercellular spaces and sinuses, which show the highest solute concentrations in this tissue, do not have a fixed orientation to the direction of freezing (Figs. 2 and 5).

A second alternative explanation for high K levels in intercellular channels is that during partial dehydration of the sections to improve image details water is lost preferentially from the large intercellular spaces, which lack an extensive organic matrix. This possibility has been ruled out by ensuring that the continuum counts are as high in the interspaces as in the cytoplasm and the lumen (see appendix in Gupta & Hall, 1979). As others have experienced (e.g. Somlyo *et al.* 1977, 1979; Dörge *et al.* 1978) we find that even complete freeze-drying at low enough temperatures preserves the spatial distribution of elements *except* in aqueous regions such as intercellular spaces and the lumen of secretory tissues (Gupta *et al.* 1978*a*). Fully dried frozen sections are not generally useful for measurements on intercellular spaces, as the ions either disappear from the spaces or their distribution becomes nonsensical, unless a substantial organic matrix is present.

Once a tissue is frozen, diffusion will continue in the solid phase, although data are not available to determine how rapidly this will occur. Some mobility for water above -120 °C is indicated by the ability of ice to undergo the phase transitions known as migratory recrystallization (Rebhun, 1972). The presence of an organic matrix probably obstructs diffusion of water and hence slows migratory recrystallization (Christensen, 1971; Gupta *et al.* 1977*a*). However, such diffusion at very low temperatures ($-80°$ to -190 °C) is likely to be very slow and restricted to nanometre distances because of an increase in viscosity by several orders of magnitude compared to that of liquid water.

Lastly, it has sometimes been suggested that local concentration gradients in our previous microprobe studies may be due to anomalous osmotic effects, produced by the inclusion of 10–20% (w/v) dextran (MW around 230000). Many macromolecules, particularly glycoproteins, are known to possess remarkable colligative properties and depress the freezing point several hundred times further than expected on the basis of their molecular weight (e.g. 'antifreeze' glycoproteins of Antarctic fish, Feeney 1974). Such natural molecules could have an important role in rectal absorption of many insects (Wall, 1977). Physiological studies have shown that 10% w/v dextran does not appreciably affect the fluid transport functions *in vitro* by Malpighian tubules of *Rhodnius* (Gupta *et al.* 1976) and by salivary glands of *Calliphora* (Gupta *et al.* 1978*a*). Civan, Hall & Gupta (1980) found that 20% dextran in Ringer does not affect the transepithelial potentials and electrical resistance in toad urinary bladders

in vitro. Forer, Gupta & Hall (1980) found that 17% dextran does not affect meiosis in the spermatocytes of the crane fly. Finally, the cryoprotective properties and physiological effects (Echlin *et al.* 1977) of high molecular weight dextrans at different concentrations have been re-investigated in our laboratory by Dr Tudor Barnard. The results show that an addition of even 25% dextran (MW 200000) to solutions: (1) does not increase the measured freezing-point depression of Ringer solution by more than a few per cent; (2) does not significantly alter the activity of K^+ measured by K-selective electrodes in KCl solutions; and (3) does not change the K concentration in the fluid secreted by the salivary glands of *Calliphora* when bathed in dextran-Ringer. The K concentration of the saliva in this tissue is a very sensitive indicator of the effective osmotic pressure of the fluid bathing the glands (Oschman & Berridge, 1970).

High K in apical fold region

It seems unlikely that the high K values detected in the apical region are an artifact due to solute migration during freezing. However, it is still worthwhile to determine whether such an artifact, if it existed, would significantly affect the calculations and the conclusions drawn in this paper. If the K located in the apical fold region in rectum V (which had the highest K values observed) were redistributed throughout the entire cell volume, this would lead to only a 7 m-equiv increase in the average cytoplasmic K value. However, the stacks make up a large portion of the cell volume. If the stack-free cytoplasm comprises only 25% of the total volume of the cell and the K in the apical fold region is redistributed within it, this would result in a 29 m-equiv increase in the average K value in stack-free cytoplasm in rectum V. This is not a large enough increase to influence significantly the conclusions drawn from comparisons of cytoplasm, stacks and intercellular spaces.

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