FLUID SECRETION BY THE MALPIGHIAN TUBULES OF THE TSETSE FLY *GLOSSINA MORSITANS*: THE EFFECTS OF OUABAIN, ETHACRYNIC ACID AND AMILORIDE

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SUMMARY

The effects of three inhibitors of sodium transport on the secretion of fluid by the Malpighian tubules of *Glossina morsitans* have been observed. The cardiac glycoside, ouabain, affects neither the rate of secretion nor the sodium concentration of the fluid secreted when isolated tubules are bathed by solutions containing a range of sodium and potassium concentrations. Secretion is inhibited, however, by ethacrynic acid and amiloride. The results confirm that fluid secretion by the Malpighian tubules of this insect is dependent on the active transport of sodium ions and show that Na^+/K^+ exchange pumps are not involved in this process.

INTRODUCTION

Recently it has been shown that the Malpighian tubules of *Glossina morsitans* generate a rapid flow of urine by the active transport of sodium ions (Gee, 1976). A speculative model of the Malpighian tubule cell was proposed and is shown in Fig. 1. It was postulated that the diuretic hormone, which controls the rate of secretion of the Malpighian tubules (Gee, 1975), stimulates rapid secretion by increasing the permeability of the basal membrane to sodium. This would allow sodium to flow into the cell down its concentration gradient, assuming that, as in other cells, the cell interior has a low sodium concentration and a high potassium concentration. It was proposed (Gee, 1976) that these intracellular levels of sodium and potassium are maintained by a Na^+/K^+ exchange pump on the basal membrane of the cell – a pump which would make no contribution to the secretion of fluid. The influx of sodium, initiated by the diuretic hormone will raise the sodium concentration inside the cell and trigger a sodium pump on the apical membrane (shown in Fig. 1). This pump secretes sodium ions and creates the local osmotic gradients necessary for the rapid secretion of fluid by insect Malpighian tubules (see Maddrell, 1971).

This cell has characteristics similar to those of other sodium-transporting epithelial cells. Therefore, in order to find out more about the mechanism of sodium transport,
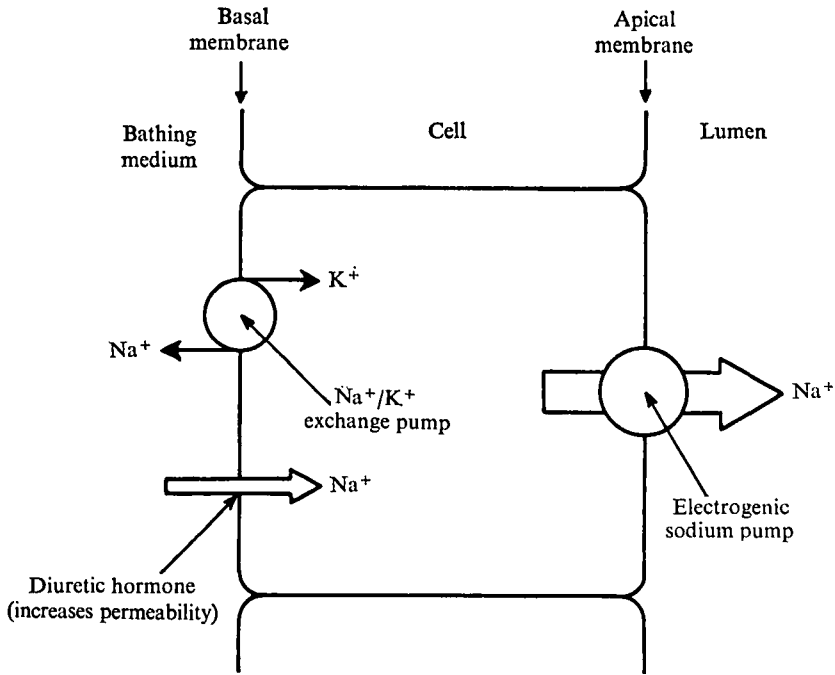
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Fig. 1. A diagrammatic representation of a Malpighian tubule cell of *G. morsitans*, showing the proposed movement of sodium ions (after Gee, 1976).

three compounds known to inhibit the movement of sodium in other cells were applied to isolated Malpighian tubules and their effects were monitored. The three compounds used were ouabain, ethacrynic acid and amiloride.

Ouabain is a cardiac glycoside which specifically inhibits a $Na^+/K^+/Mg^{2+}$-activated ATPase. By inhibiting this enzyme it prevents the action of pumps found in a variety of cells (see Glynn, 1964), which couple the extrusion of sodium with the influx of potassium. It was thought that this compound might inhibit the exchange pump situated on the basal membrane in the model shown in Fig. 1.

Ethacrynic acid has been shown to inhibit that part of cellular sodium transport which does not rely on the exchange of sodium for potassium and is not inhibited by ouabain. Examples of cells in which it inhibits the movement of sodium are kidney proximal tubular cells (Whittembury, 1968; Whittembury & Fishman, 1969), cells of the frog sartorius muscle (Erlij & Leblanc, 1971) and human red blood cells (Lubowitz & Whittam, 1969). This compound might provide information about the sodium pump shown on the apical cell membrane in Fig. 1.

Amiloride is an aminopyrazine compound which inhibits sodium transport by blocking the entry of sodium into the transporting cells. Its effect has been demonstrated on sodium-transporting epithelia such as the toad bladder (Bentley, 1968) and the frog skin (Nagel & Dörge, 1970), and it was hoped that this compound might show whether rapid fluid secretion was dependent on high permeability to sodium at the basal cell membrane.

MATERIALS AND METHODS

Experimental method

Teneral adult *Glossina morsitans* were taken from the self-supporting colony maintained at Langford. Malpighian tubules from these insects were set up as *in vitro* preparations by the method previously described (Gee, 1976). They were isolated into drops of Ringer's solution containing 10^{-3} M 3',5'-cyclic AMP, which stimulates rapid secretion by *Glossina* tubules (Gee, 1976). These initial drops were replaced by drops containing ouabain, ethacrynic acid or amiloride dissolved in Ringer containing 10^{-3} M cyclic AMP. The rate of secretion in the presence of these compounds was expressed as a percentage of the initial rate of secretion in Ringer containing cyclic AMP alone.

The experiments were performed at room temperature, 19–22 °C.

Experimental solutions

The basic Ringer's solution had the following composition (mM): NaCl, 120; KCl, 10; glucose, 20; malic acid, 3; citric acid, 2; $MgCl_2.6H_2O$, 2; $NaH_2PO_4.2H_2O$, 1·5; $CaCl_2.6H_2O$, 2. The pH was adjusted to 7 with NaOH (bringing the total sodium concentration to 140 mM) and phenol red was added to keep a constant check on the pH.

Cyclic AMP, ouabain and ethacrynic acid were all readily soluble in Ringer's solution at the concentrations used in this investigation. Amiloride, however, was only partially soluble and had to be first dissolved in distilled water, to which a sufficient quantity of concentrated Ringer was then added to bring both to their correct concentrations. At concentrations above 2×10^{-3} M solutions of amiloride in Ringer were not stable at room temperature.

3',5'-cyclic AMP was obtained from Boehringer, Mannheim; ouabain (strophanthin-G) from Sigma and ethacrynic acid and amiloride from Merck, Sharp & Dohme.

RESULTS

The effect of ouabain

In a previous investigation of fluid secretion by the Malpighian tubules of *G. morsitans*, ouabain was applied to tubules secreting in the basic Ringer (140 mM-Na, 10 mM-K) and its effect was monitored for 1 h. During that time it caused no alteration in the rate of secretion (Gee, 1976). In the present series of experiments the period monitored was extended to 4 h so that any slow-acting effects of ouabain might be observed. The effect of ouabain on tubules secreting in Ringers containing different sodium and potassium concentrations was also tested. In each experiment the rate of secretion of tubules isolated into drops of basic Ringer containing 10^{-3} M cyclic AMP was measured, after which the tubules were transferred to drops of Ringer with different sodium and potassium concentrations and containing 10^{-3} M cyclic AMP and 10^{-3} M ouabain. Control tubules were transferred to similar drops from which ouabain was omitted.

In a Ringer containing 145 mM-Na, 5 mM-K the rate of secretion of tubules in the presence of ouabain fell no more rapidly over a period of 4 h than did the rate
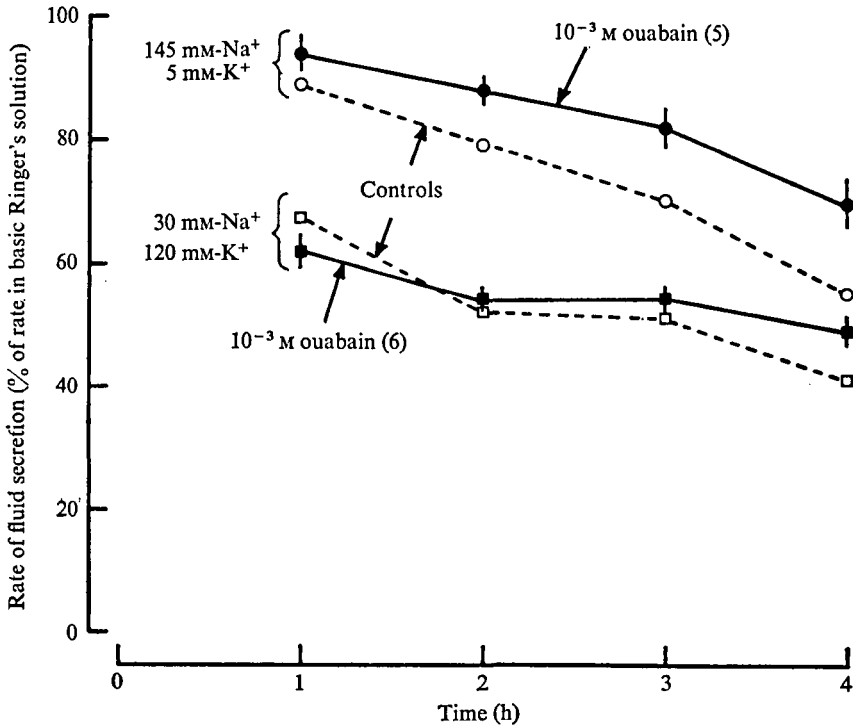
Fig. 2. The effect of ouabain on the rate of fluid secretion. The values in the presence of ouabain are means, the number of experiments are shown in parentheses and the vertical lines represent the extent of twice the standard error of the mean. The values of the controls are means of two experiments.

of secretion of the control tubules (Fig. 2). The concentration of sodium in the secreted fluid was measured and was found to be unaffected by the presence of ouabain (Table 1).

In the complete absence of potassium from the bathing medium, 10^{-3} M ouabain did not reduce the temporary acceleration of fluid secretion elicited by cyclic AMP (Fig. 3), nor did it affect the sodium concentration of the secreted fluid (Table 1).

The rate of fluid secretion by tubules transferred to drops containing 30 mM-Na, 120 mM-K was lower than in the basic Ringer since the rate of secretion is dependent on the sodium concentration of the bathing medium (Gee, 1976). However, the presence of ouabain did not cause a greater decrease in either the rate of secretion (Fig. 2) or the concentration of sodium in the secreted fluid (Table 1) in comparison with the control tubules.

The effect of ethacrynic acid

The results of two typical experiments showing the effect of ethacrynic acid on the rate of fluid secretion by isolated tubules are reproduced in Fig. 4. 10^{-3} M ethacrynic acid rapidly reduced the rate of secretion to zero. This effect was not reversible, for even after repeated washing in Ringer's solution the tubule did not recover its secretory ability when returned to Ringer containing cyclic AMP. 10^{-5} M ethacrynic acid, on the other hand, caused only partial inhibition of fluid secretion.
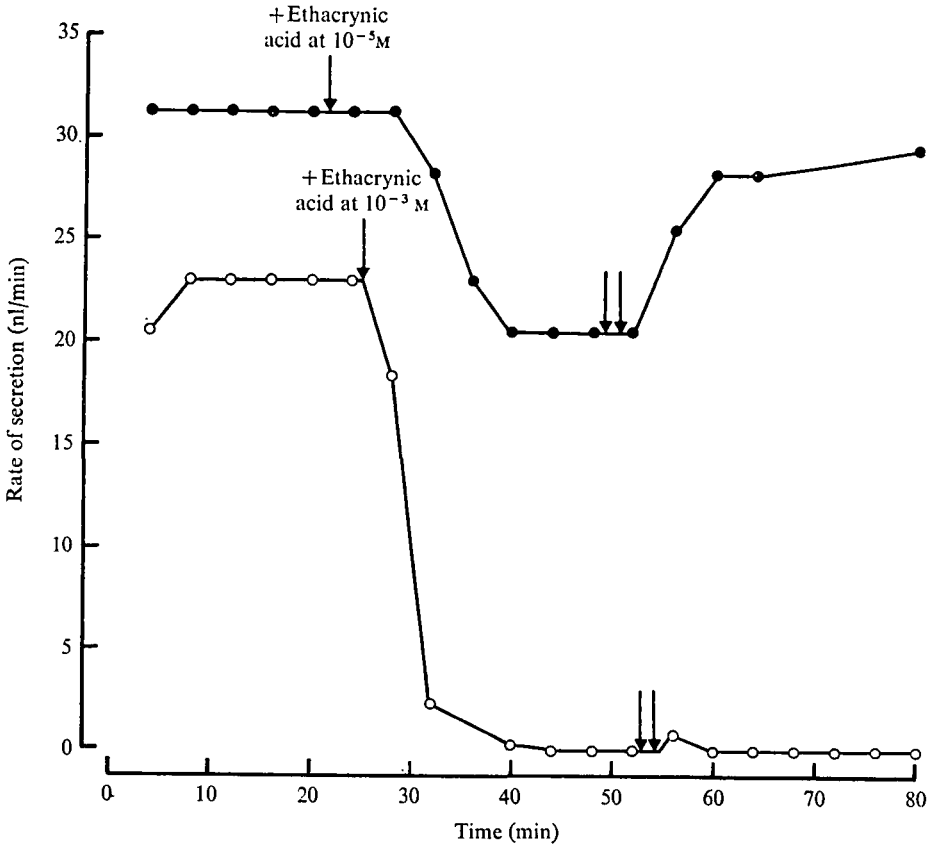
Fig. 4. The effect of ethacrynic acid on the rate of secretion by Malpighian tubules of *Glossina* secreting in the presence of 10^{-3} M cyclic AMP. The single arrows mark the times at which ethacrynic acid was added to give the concentrations shown. At the times indicated by the double arrows the bathing drops were removed, the tubules were washed with Ringer's solution and drops of fresh Ringer containing 10^{-3} M cyclic AMP were added.

The effect of amiloride

The results of a typical experiment to show the effect of amiloride on the rate of fluid secretion are plotted in Fig. 6. 2×10^{-3} M amiloride caused total inhibition of fluid secretion. However, unlike the effect of ethacrynic acid, inhibition by amiloride was rapidly and totally reversible when the tubule was returned to a drop of Ringer's solution containing cyclic AMP. Fig. 7 shows the dose/response curve for the effect of amiloride, a 50 % inhibition of fluid secretion occurring at a concentration of 5×10^{-5} M.

DISCUSSION

The results of the present series of experiments confirm the previous conclusion (Gee, 1976) that a ouabain-sensitive Na^+/K^+ exchange pump is not responsible for the active transport of sodium which generates the movement of fluid during rapid secretion by the Malpighian tubules of *Glossina*. Even when the sodium concentration of the bathing medium was reduced to a level which restricted the rate of fluid
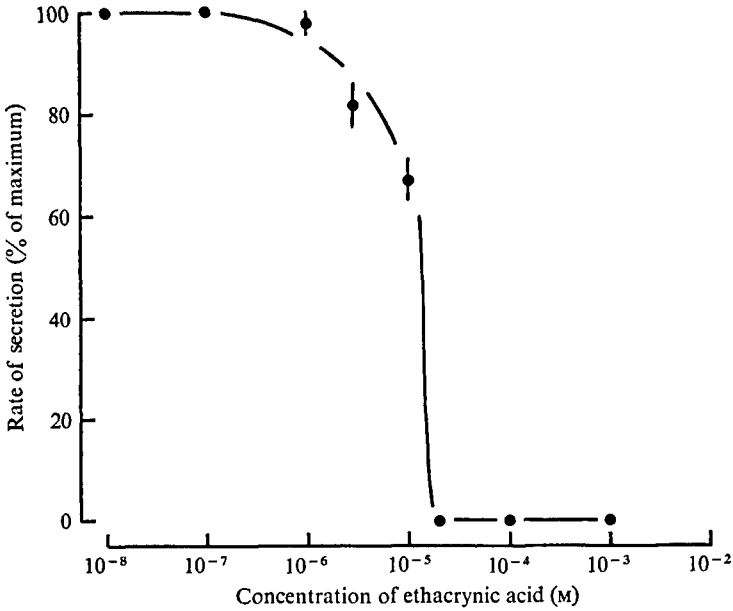
Fig. 5. Dose/response curve for the inhibition of secretion by the Malpighian tubules of *Glossina* by ethacrynic acid. Each point is the mean of six determinations, the vertical lines show the extent of twice the standard error of the mean.
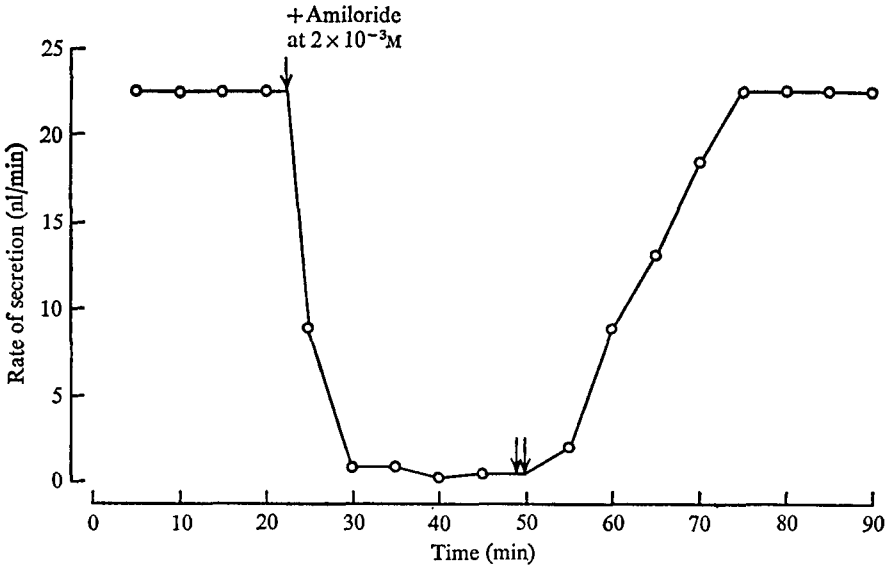

Fig. 6. The effect of amiloride on the rate of secretion of *Glossina* tubules secreting in the presence of 10^{-3} M cyclic AMP. Amiloride was added at the time indicated by the single arrow to give the concentration shown. The double arrow marks the time at which the bathing drop was removed, the tubule was washed with Ringer and a drop of fresh Ringer containing 10^{-3} M cyclic AMP was added.
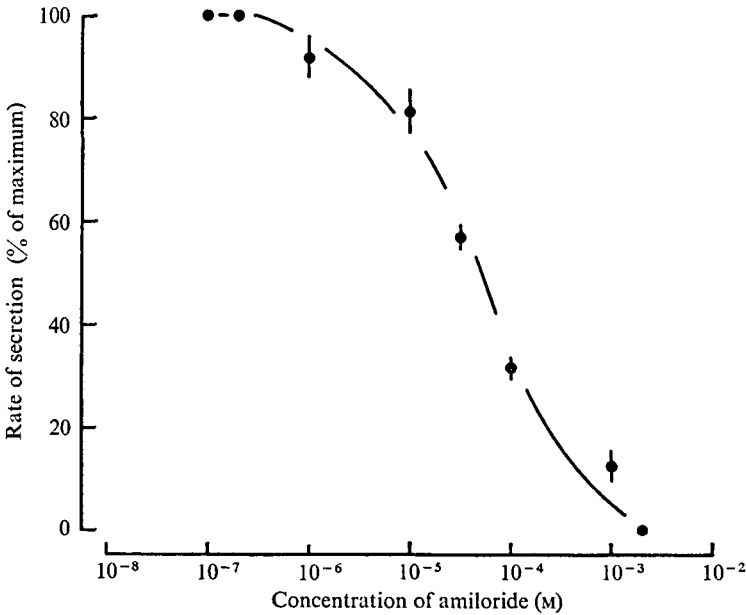
Fig. 7. Dose/response curve for the inhibition of secretion by the Malpighian tubules of *Glossina* by amiloride. Each point is the mean of six determinations, the vertical lines represent the extent of twice the standard error of the mean.

production, ouabain caused no further decrease in the rate of secretion. It is therefore clear that the inhibition of diuresis in *G. morsitans* by ouabain ingested in a saline solution, which was observed by Gooding (1975), must have been due to the action of this compound at a site other than the Malpighian tubules. The situation in *Glossina* is somewhat different to that in other insects, since sodium rather than potassium is transported by the Malpighian tubules in order to generate fluid secretion (Gee, 1976). Nevertheless, in *Calliphora* (Berridge, 1968), *Rhodnius* (Maddrell, 1969) and *Carausius* (Pilcher, 1970) secretion by the Malpighian tubules is similarly unaffected by ouabain. Recently ouabain has been shown to reduce the rate of fluid secretion by the Malpighian tubules of *Locusta* (Anstee & Bell, 1975), though it is not clear how a Na^+/K^+ exchange pump would produce the net movement of ions required to establish the local osmotic gradients necessary for fluid secretion (see Maddrell, 1971).

It was previously suggested (Gee, 1976) that besides the sodium pump on the apical membrane a separate Na^+/K^+ exchange pump was present on the basal membrane of the tubule cell (as shown in Fig. 1). The function of this pump would be to maintain a high potassium concentration and a low sodium concentration within the cell and it would therefore not be involved in the net transport of sodium across the cell or the secretion of fluid. In order to explain why fluid secretion was inhibited by the removal of potassium from the bathing medium, it was postulated (Gee, 1976) that in the absence of external potassium this exchange pump was unable to maintain the high intracellular potassium concentration and the resulting loss of potassium from the cell led to the cessation of fluid secretion. The presence of a ouabain-sensitive exchange pump with the function of maintaining a high

intracellular potassium concentration would be most readily demonstrated if the tubules were bathed by solutions of low potassium concentrations. Inhibition of the exchange pump by ouabain would then most rapidly lead to the loss of potassium from the cells and a cessation of secretion. However, in the present series of experiments, with the potassium concentration of the bathing medium reduced by half (to 5 mM), ouabain caused no decrease in the rate of secretion over a period of 4 h, and even in the total absence of potassium, ouabain did not diminish the secretory response. Measurement of the sodium concentration of the secreted fluid indicated that sodium remained the actively transported ion throughout.

The results of this investigation demonstrate that the functioning of a Na^+/K^+ exchange pump on the basal membrane is not necessary for rapid secretion to continue. However, the presence of such a pump, the function of which may be to maintain the intracellular ion concentrations of the resting cell, cannot be ruled out in the absence of data on the resting cell.

It is clear that although the Malpighian tubules of *Glossina* rely on the active transport of sodium ions to generate the flow of urine, sodium is not transported by a ouabain-sensitive Na^+/K^+ exchange pump. A possible alternative would be for the cell to use an electrogenic sodium pump. The transport of sodium out of the cell would then lead to an increase in the potential across the apical membrane, unless the movement of sodium were linked to the movement of an anion such as chloride in the same direction. A pump of this nature is found in the cells of the kidney proximal tubule (Whittembury, 1968; Whittembury & Fishman, 1969). Its characteristics are that sodium is extruded together with chloride and water without being exchanged for potassium; the transport of sodium may occur in the presence of ouabain but is inhibited by ethacrynic acid.

As we have seen, the secretion of fluid by the Malpighian tubules of *Glossina* is inhibited by ethacrynic acid, and since this compound causes the complete inhibition of fluid secretion, pumps sensitive to ethacrynic acid must be able to account for the total transport of sodium ions and the secretion of fluid. This fact adds further weight to the conclusion that Na^+/K^+ exchange pumps are not involved in the secretion of fluid, for such pumps are insensitive to ethacrynic acid (Whittembury & Fishman, 1969) and would be able to maintain a background level of secretion in the presence of this compound. The sodium pump may be situated on the apical membrane of the tubule cell, as proposed in Fig. 1, but in order to confirm this the potential difference across the apical and basal membranes of the cell must be established.

The sensitivity of the tubules to amiloride indicates that they require the basal membrane of the cell to be permeable to sodium ions for fluid secretion to occur. In experiments on the toad bladder (Bentley, 1968) and frog skin (Nagel & Dörge, 1970) it has been demonstrated that the effect of amiloride is to restrict the entry of sodium into the cell and thereby reduce the intracellular pool of sodium available to the sodium pump on the opposite cell membrane. As with the isolated frog skin (Salako & Smith, 1969), the inhibitory effect of amiloride can be reversed by washing the preparation with fresh Ringer's solution, indicating that the permeability barrier to sodium is situated at the basal membrane of the cell. This membrane must therefore control the access of sodium to the pumps on the apical membrane.

This investigation has provided further information about the nature of the secretory mechanism of the Malpighian tubules of *Glossina*. It has confirmed that sodium is the actively transported ion during rapid secretion, for disruption of sodium transport by ethacrynic acid and amiloride prevents fluid secretion. The investigation has also shown that pharmacological agents which affect sodium transport in vertebrate epithelia have similar effects on sodium transport by the Malpighian tubules of *Glossina* – indicating that a common mechanism of sodium transport might exist at the subcellular level.

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