J. exp. Biol. (1979), 78, 213–223
With 7 figures
Printed in Great Britain

BATHING SOLUTION TONICITY AND POTASSIUM TRANSPORT BY THE MIDGUT OF THE TOBACCO HORNWORM *MANDUCA SEXTA*

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(*Received* 24 *April* 1978)

SUMMARY

Potassium transport by the isolated midgut of *Manduca* larvae, as measured by the short circuit current, is inhibited by substitution of small organic solutes (M.W. < 340) for the sucrose normally included in bathing solution formulated for this tissue. Other solutes of molecular weight equal to or greater than sucrose are essentially as effective as sucrose in promoting the short circuit current. Equilibration of midgut in solutions containing the small solute mannitol results in a decrease in the dry weight/wet weight ratio of the tissue, suggesting that the small solutes can penetrate into areas of the tissue which are not accessible to sucrose. Histological studies suggest that sites of swelling in the presence of mannitol include both cytoplasm and goblet cell lumen. The inhibition of the short circuit current is rapidly reversible on return to bathing solution containing sucrose or another large solute. The effect of small solutes probably does not involve compromise of the energy source for potassium transport since oxygen uptake is unchanged in the presence of a small solute.

INTRODUCTION

The midgut of the tobacco hornworm *Manduca sexta* has been shown to actively transport potassium from blood to lumen by a mechanism which resembles that of other lepidopteran larvae in that the bulk of the short circuit current can be accounted for by potassium transport, and in that the active potassium transport is oxygen-dependent (Moffett, unpublished observations: Blankemeyer & Harvey, 1977). In the silkworm *Hyalophora cecropia* the active potassium transport has been held to be electrogenic (Harvey, Haskell & Nedergaard, 1968) and an active step for potassium movement has been located at the apical membrane of goblet cells (Wood, Farrand & Harvey, 1969; Blankemeyer & Harvey, 1977).

Bathing solutions formulated for the isolated midgut of *Hyalophora cecropia* contain sucrose as an osmotic effector and this sucrose has been reported to be necessary for continued potassium transport (Harvey & Zerahn, 1972; Zerahn, 1977). The present study investigates the effects of replacing the sucrose with uncharged solutes of smaller molecular weight. A preliminary report has been made (Moffett, 1976).

MATERIALS AND METHODS

Larvae of *Manduca sexta* L. were raised at 27° on a 17 h light/7 h dark cycle, using the artificial diet of Yamamoto (1969).

Midguts were rapidly excised from fifth instar larvae which had been anaesthetized by packing them in crushed ice for 1 h. For measurement of the transepithelial electrical potential (TEP) and short circuit current (SCC), the excised gut was mounted as a flat sheet between two half-chambers which were provided with oxygenation and with electrodes for measuring TEP and passing SCC. The area of the aperture between the two chambers was 0·22 cm^2 and SCC values given are for this nominal area of tissue. In the studies we report here, a two-electrode system was used for measuring TEP, and the SCC was not corrected for the contribution of bathing solution resistance between the voltage electrodes; thus the SCC is a relative but not quantitative measure of the net ion transport of the tissue. However, all test solutions used in any experimental series were formulated to be of similar electrical resistivity to ensure that the error in measuring the SCC would be constant.

For measurement of oxygen consumption, excised midguts were shorn of tracheae and Malpighian tubules and were cut into longitudinal strips. The strips were placed in flasks of a Gilson differential respirometer. The flasks were maintained at 27 °C, gassed with 100% oxygen, and agitated at a rate exceeding that found to be necessary for optimal oxygen delivery in preliminary studies. Stable values of oxygen uptake were recorded for at least one hour. At the end of the experiment the strips were removed from the flasks, blotted by a standardized method, rapidly weighed to determine wet weight, and dried to constant weight to determine dry weight.

For measurements of the dry weight/wet weight ratio and potassium content of midgut, strips similar to those used in respiration studies were equilibrated for one hour in oxygenated 32 K-S bathing solution (see below), and subsequently were transferred to continuously oxygenated beakers of test solution, where they remained for an additional hour. Thereafter tissue wet weight and dry weight were determined as in the respiration studies. The dried tissue was dissolved in concentrated nitric acid and the resulting solution was diluted to a known volume. Potassium concentration of the solution was determined by atomic absorption spectrophotometry, and net potassium concentration of the tissue was calculated from this value and the difference between wet and dry weights.

The basic bathing solutions used in these studies are like those used in studies of the isolated midgut of *Hyalophora cecropia* (Zerahn, 1977); this is appropriate since the blood composition of the two species is similar (Jungreis, Jatlow & Wyatt, 1973). The bathing solutions were composed as follows (in mM):

32 K-S: KCl, 30; $KHCO_3$, 2; $CaCl_2$, 5; $MgCl_2$, 5; Tris HCl, 5 (pH = 8·0); sucrose, 166.

70 K-S: KCl, 68; $KHCO_3$, 2; $CaCl_2$, 5; $MgCl_2$, 5; Tris HCl, 5 (pH = 8·0); sucrose, 90.

Other solutions are described where appropriate.

For histological studies, midgut strips were equilibrated for 1 h in oxygenated 32 K-S, transferred to either 32 K-S or test solution for 1 h, and fixed in 1% osmium

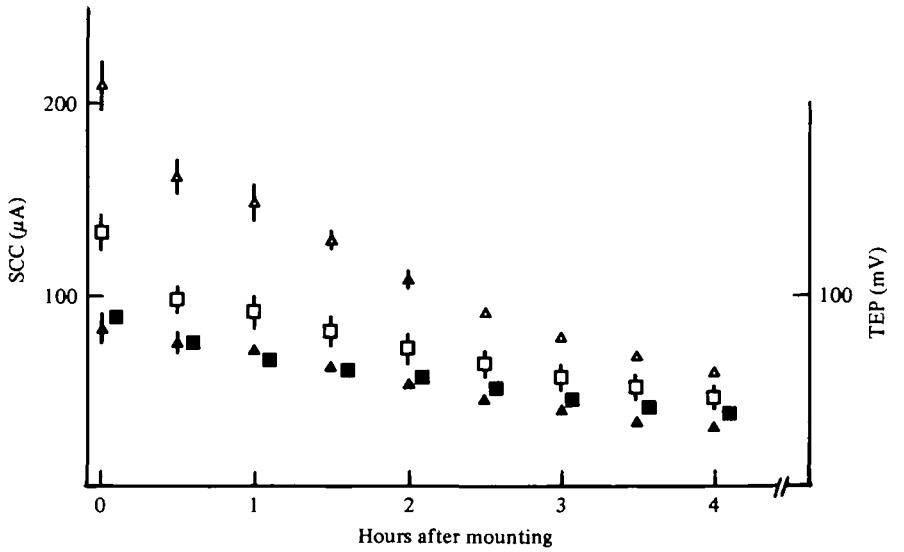

Fig. 1. SCC (triangles) and TEP (squares) of isolated midgut preparations bathed in either 32 K-S (filled symbols) or 70 K-S (open symbols); the vertical bars indicate ± 1 S.E. for five experiments. In the absence of a vertical bar the standard error is less than the height of the symbol.

tetroxide in bathing solution for 2 h. After fixation, tissue was embedded in Epon and 1 μm sections were cut. Sections were stained with toluidine blue and examined by light microscopy.

RESULTS

1. *Correction for decay of SCC with time*

Fig. 1 shows the time course of SCC and TEP of midgut preparations bathed in 32 K-S and in 70 K-S. As with other lepidopteran midguts (Harvey & Nedergaard, 1964; Wood, 1972; Giordana & Sacchi, 1977), the SCC and TEP diminish with time. In most cases in the present studies it was desirable to express the SCC developed by the isolated midgut when bathed in a test solution as a percentage of that developed in a standard solution. Generally measurements of SCC in test solutions were preceded and followed by measurements in standard solution. The short circuit decay curves shown in Fig. 1 were used to determine the value of SCC to be expected if the tissue had remained in standard solution, and the steady SCC in test solution was expressed as a percentage of this value. Results were discarded if upon return to standard bathing solution the SCC did not recover to within ± 1 standard deviation of that predicted by the decay curve. In this way the intrinsic decay of the SCC with time was compensated for and experiments which resulted in irreversible damage to the tissue were eliminated.

2. *The effect of replacing sucrose with mannitol*

Fig. 2 shows the effect on SCC and TEP of replacing sucrose in the 32 K-S bathing solution with mannitol (32 K-M solution). For comparison, the normal time

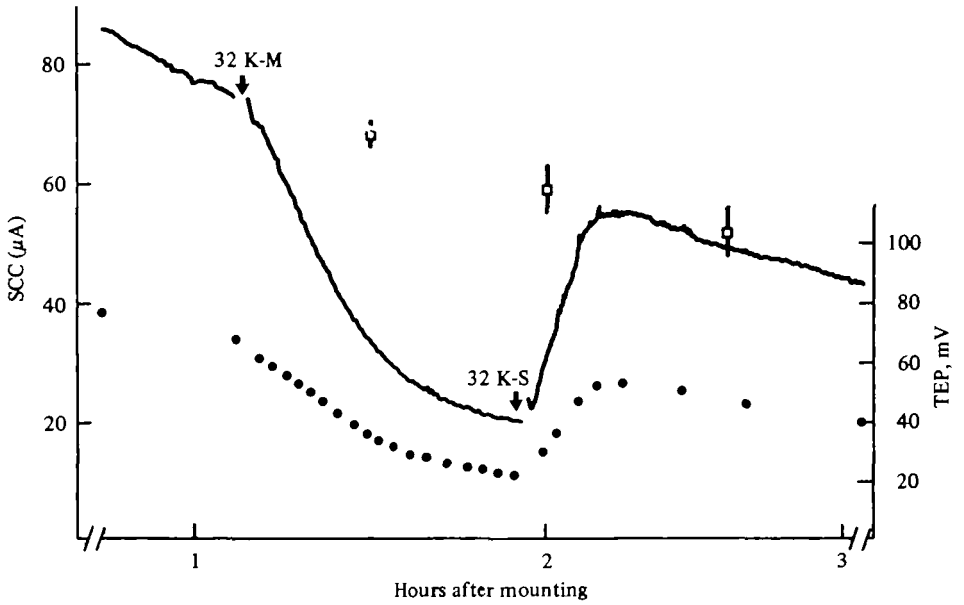

Fig. 2. SCC (continuous trace) and TEP (dotted line) of a midgut equilibrated in 32 K-S and transferred to 32 K-M. For assessment of recovery, appropriate points from Fig. 1 are also shown (see text for Fig. 1).

course of the SCC as measured in 32 K-S (data from Fig. 1) is also shown. The experiment shown is typical of seven such experiments. In some cases the TEP and SCC fall almost to zero; in others low but steady values of SCC and TEP can be maintained in 32 K-M. The mean minimum value of SCC in 32 K-M in the seven experiments was 36% (S.E. = 3). Upon return to 32 K-S, recovery is rapid and complete when the intrinsic decay of the SCC and TEP are taken into account.

In experiments in which 32 K-M was substituted for 32 K-S only on the lumen side of the gut, the resulting SCC was reduced to 41% (S.E. = 5, $N = 7$) of the expected value with 32 K-S on both sides; in similar experiments in which 32 K-M replaced 32 K-S on the blood side only, the resulting SCC was 66% (S.E. = 10, $N = 7$) of the expected value under control conditions. This result suggests that both faces of the midgut are susceptible to the effects of bathing solution composition.

3. *Effect of substituting other organic solutes for sucrose*

Fig. 3 shows the relative ability of mannitol and other organic solutes to support a SCC when substituted for sucrose in the 32 K-S formula, as plotted against the molecular weight of the substituted compounds. There is a clear general relationship between the molecular weight of the substituted compounds and their ability to support transport, with optimal effect shown by compounds whose molecular weight is equal to or greater than that of sucrose. It is clear, however, that molecular weight is not an absolute predictor of the effectiveness of a compound in protecting the short circuit current. It may be appropriate to distinguish two groups of compounds, with fructose, melibiose, maltose, trehalose, cellobiose, lactose and sucrose being more effective with respect to molecular weight than glucose, mannitol, inositol, poly-

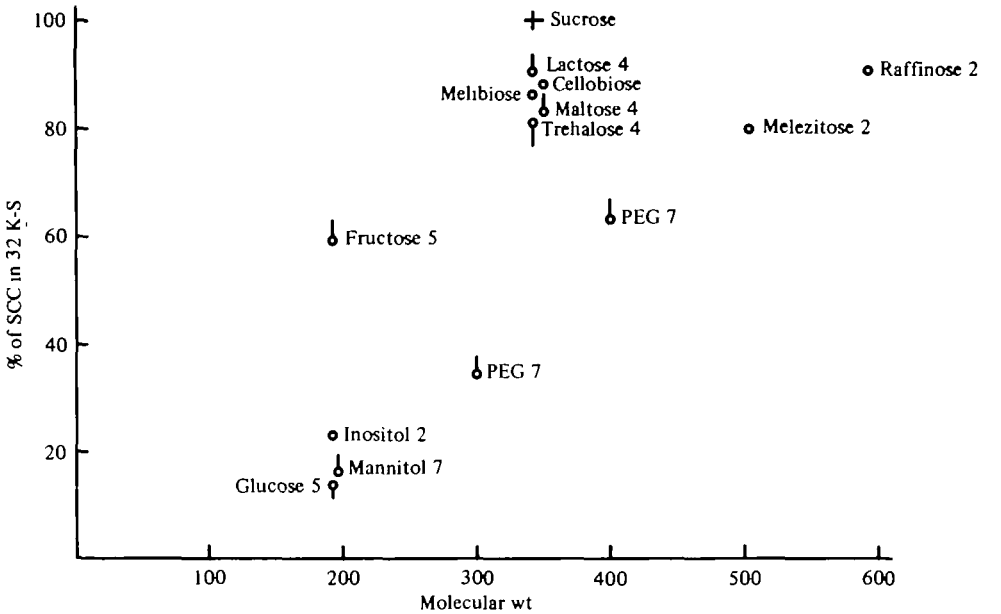

Fig. 3. The ability of various solutes to support the SCC when substituted for sucrose in the 32 K-S solution. The number of experiments is given next to each point. Vertical bars indicate 1 S.E. For points represented by fewer than four experiments, a simple mean is given. 'PEG' stands for polyethylene glycol, of which two different molecular weights were used.

ethylene glycol, melezitose and raffinose. The reason for this difference is not clear, since each group contains both metabolizable and non-metabolizable compounds.

4. *Oxidative metabolism in 32 K-S and 32 K-M*

The oxygen uptake of six midgut strips equilibrated in 32 K-S solution was 73·9 (S.E. = 3) μequiv oxygen/g dry weight^{-1} min^{-1}; the value for six corresponding strips equilibrated in 32 K-M was 80·8 (S.E. = 6·9) μequiv g dry weight^{-1} min^{-1}. This result shows that the inhibition of potassium transport that occurs when mannitol is substituted for sucrose is not attributable to inhibition of the pathway of oxidative energy metabolism upon which the ion transport depends (Harvey, Haskell & Zerahn, 1967).

5. *Effect of potassium concentration on inhibition of SCC by substituting mannitol for sucrose*

Fig. 4 shows the effect on SCC of substituting mannitol for part or all of the sucrose in solutions containing either 32 m-equiv K^+/litre or 7 m-equiv K^+/litre. The latter solutions contain 25 m-equiv Na^+/litre so that all solutions in this experiment are of closely similar ionic strength and electrical conductivity. Note that in both cases the magnitude of the SCC is in direct proportion to the sucrose concentration of the medium, so that the effect of substituting mannitol for sucrose is a graded inhibition of potassium transport.

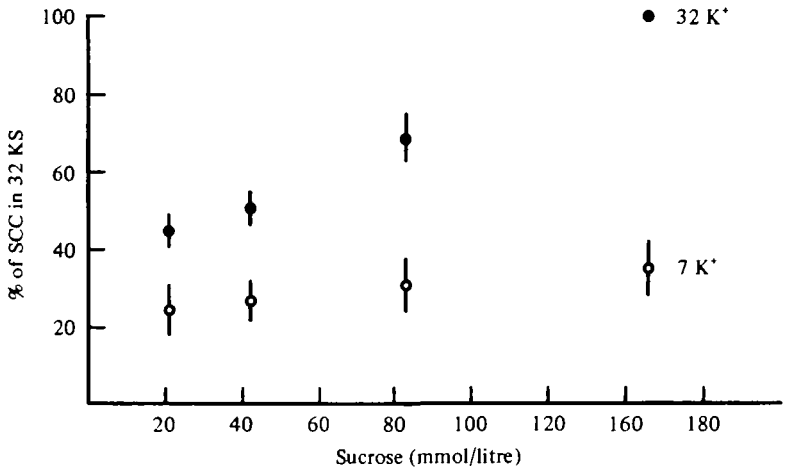

Fig. 4. Relationship of sucrose concentration to SCC at normal (32 m-equiv/l) and low (7 m-equiv/l) potassium concentrations. All values are normalized to the value in normal 32 K-S solution. Mannitol was substituted for sucrose to obtain the lower sucrose concentrations, and NaCl was substituted for KCl to obtain 7 m-equiv K^+/l bathing solution.

6. *Effect of sucrose concentration on the relationship between potassium concentration and short circuit current*

Active ion transport systems frequently display saturation kinetics; that is, the rate of active ion movement depends on the concentration of transported ion at low concentrations and becomes independent of it at higher concentrations. This quality has been taken as evidence of chemical mediation of such transport processes (cf. Kirschner, 1970), and the range of concentrations over which the transport rate is dependent on ion concentration has been held to reflect the affinity of the transport system for its ionic substrate (Potts & Parry, 1964). Nedergaard & Harvey (1968) found that saturation of the potassium transport system of the *Hyalophora cecropia* midgut did not occur even at 60 m-equiv K^+/l, a concentration about twice that of the normal blood of the animal. The experiments to be described in this section are designed to determine the relationship between SCC and potassium concentration in *Manduca* midgut and the effect on this relationship of replacing sucrose with mannitol.

For these experiments the basic bathing solution was 70 K-S. Test solutions with lower sucrose concentrations were obtained by substituting mannitol for sucrose; lower potassium concentrations were obtained by substituting NaCl for KCl. Fig. 5 shows the relationship between potassium concentration and SCC at three constant sucrose concentrations. Note first that at the highest sucrose concentration (90 mM) the transport mechanism is strongly stimulated by increasing potassium concentration and does not display saturation kinetics with regard to potassium over this range of concentrations. At lower sucrose concentrations increases in potassium concentration fail to stimulate potassium transport (10 mM sucrose) or even increase the inhibition of the SCC (0 mM sucrose). This result suggests that at low sucrose concentration increasing the potassium concentration in the external medium fails to result in increased availability of potassium to the apical pump; alternatively, there may be

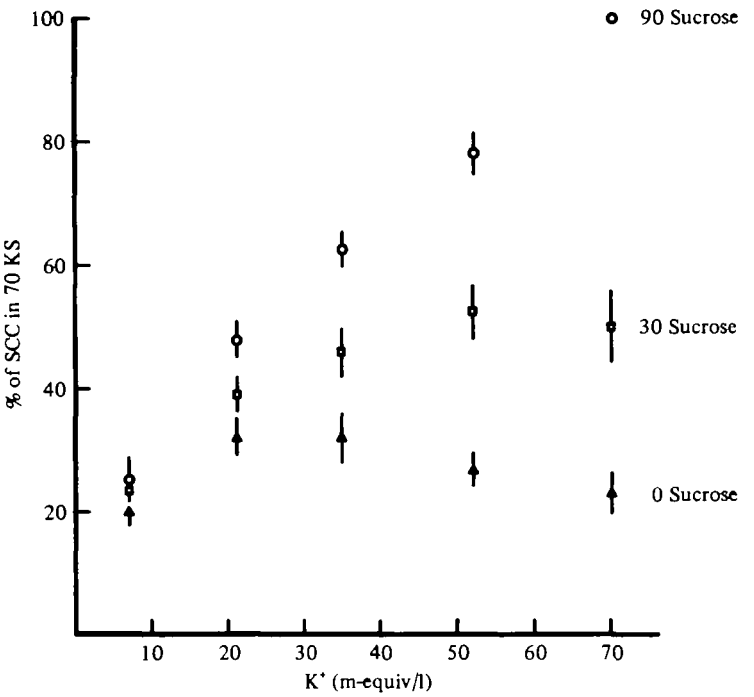

Fig. 5. Relationship of SCC to [K^+] at three different sucrose concentrations. Mannitol was substituted for sucrose and NaCl for KCl so that nominal osmoticity and ionic strength of all solutions were the same.

an effect on the tissue or the pump such that increasing potassium concentration inhibits the pump since the kinetics of the transport system with low sucrose are reminiscent of those of substrate inhibition. It should be noted that at the lowest potassium concentrations (highest sodium concentrations) in Fig. 5 there may be some active sodium transport as demonstrated by Harvey & Zerahn (1971, 1972); this may explain the fact that the curves do not readily extrapolate to zero SCC at zero potassium concentration.

7. *Effect of mannitol substitution on tissue water and potassium content*

Fig. 6. shows the dry weight/wet weight ratio and potassium/tissue water ratio of midgut strips equilibrated in solutions containing 32 m-equiv K^+/l and either 166 mM sucrose, 83 mM sucrose, or no sucrose, with the latter two solutions containing mannitol in appropriate concentration to keep the nominal osmoticity of the three solutions identical. These experiments show that the effect of replacing sucrose with mannitol is to increase the net tissue water with a proportionate decrease in the net tissue potassium concentration; thus the mannitol solutions are nominally isomotic with 32 K-S but functionally hypotonic as compared to it. It should be noted that values reported here for net tissue K^+ are substantially less than intracellular K^+ values recently reported from other species (Zerahn, 1977; Giordana & Sacchi, 1978); the difference seems unlikely to be due exclusively to the error arising from extracellular fluid, but may also be due to potassium loss during the 2 h of incubation in the present studies.

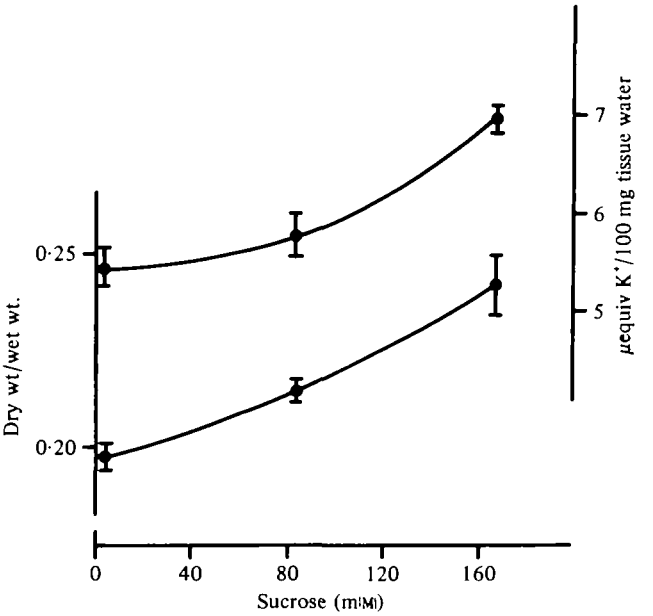

Fig. 6. Dry wt/wet wt ratio (lower curve), and net [K+] (upper curve) of midgut strips bathed in 32 K-S, 32 K-M or a solution containing 83 mM sucrose, 83 mM mannitol, and ionic solutes as in 32 K-S.

8. *Effect of 32 K-M on midgut histology*

Some information about the site of fluid accumulation in the absence of sucrose can be gained by examination of the microscopic appearance of midgut tissues after equilibration in 32 K-M.

Fig. 7A shows midgut tissue which was maintained in oxygenated 32 K-S for 2 h after removal from the animal. Goblet cells are readily distinguished from surrounding columnar cells by their prominent lumens and their lack of apical brush borders. Fig. 7B shows tissue (from the same region of the midgut of the same animal as that shown in Fig. 7A) that was equilibrated in oxygenated 32 K-S for 1 h and then transferred to oxygenated 32 K-M for an additional hour. The 1 h treatment in 32 K-M is comparable to the time needed for maximum inhibition of SCC in 32 K-M (see Fig. 2). In Fig. 7B evidence of cellular swelling may be seen in both cell types in apparent cytoplasmic bulges on the apical side of the tissue and there is less dense staining of cytoplasm than in the control tissue of Fig. 7A. Note also the larger size and swollen appearance of goblet cell lumens in Fig. 7B as compared to those in Fig. 7A. These results suggest that the increase in tissue water content resulting when mannitol is substituted for sucrose involves both cytoplasm and goblet cell matrix. Swelling of the goblet cell lumens suggests that free exchange of fluid between the goblet lumen and the exterior does not occur.

DISCUSSION

Harvey *et al.* (1967) showed that the midgut of *Hyalophora cecropia* is unusual among transporting epithelia in that while potassium transport depends on oxidative

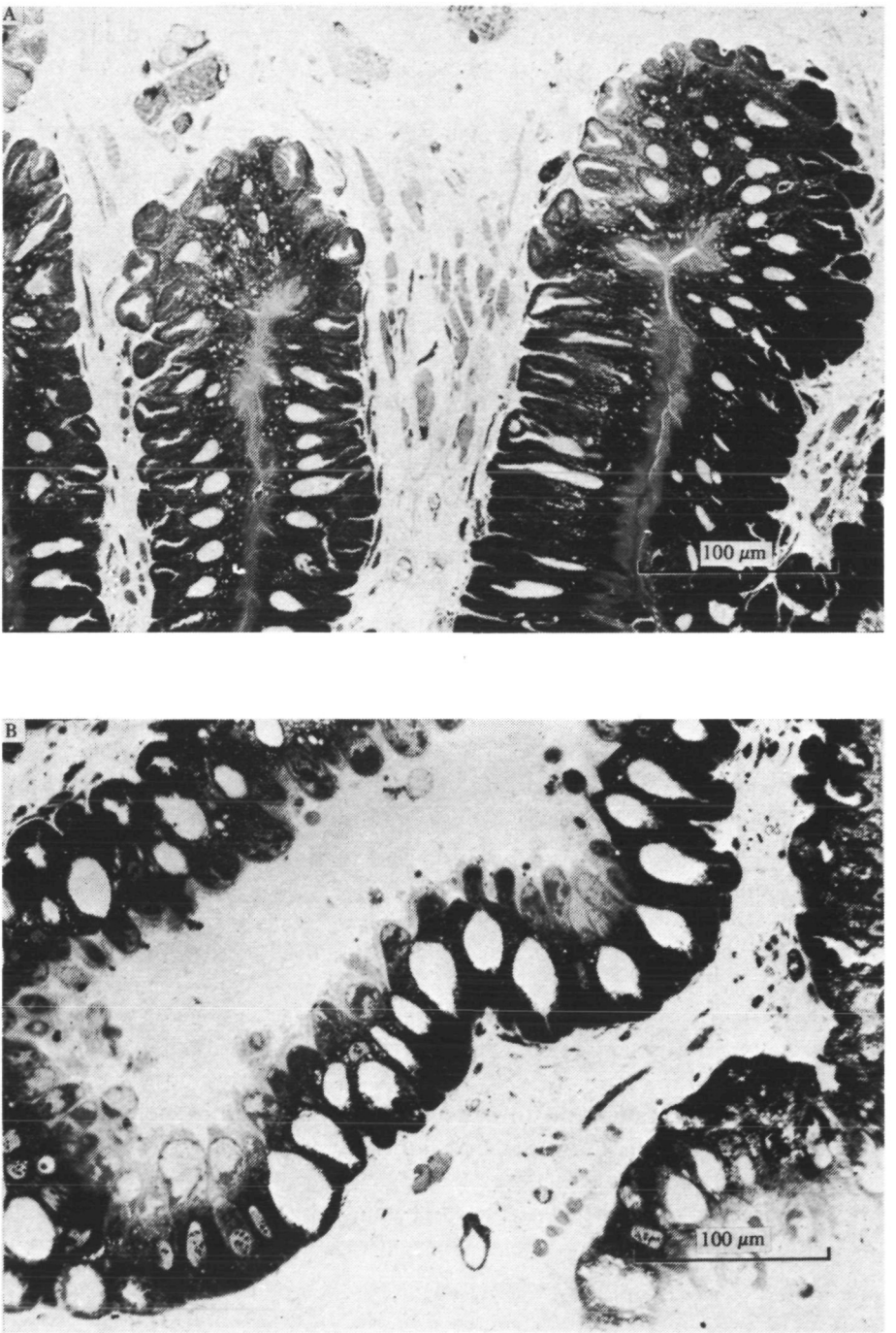

Fig. 7. (A) Section of midgut equilibrated in 32 K-S (see Methods). The tissue is folded so that the lumen side is towards the centre of each of the two folds shown. Many goblet cells are visible in sagittal and cross-section. (B) Section of midgut equilibrated in 32 K-M. The lumen side of the tissue is towards the inside of the fold. Note the swollen goblet lumens and cytoplasmic bulges on the lumen side of both epithelial cell types.

energy metabolism and must require a significant fraction of the total energy budget of the tissue, no change in oxygen consumption results from imposed changes in the rate of potassium transport. The present studies suggest that the inhibitory effect of bathing solution hypotonicity on the SCC and TEP does not involve inhibition of oxidative respiration, and add to the evidence that inhibition of potassium transport does not result in inhibition of oxidative respiration.

The increase in tissue water content which results from replacing sucrose with mannitol can be understood if mannitol and other solutes of similar size can enter tissue space inaccessible to sucrose and larger solutes, as has been suggested in other tissues (Biber, Cruz & Curran, 1972; Nellans & Schultz, 1976). The resulting inhibition of potassium transport might be due to simple dilution of the potassium transport pool, or to structural rearrangements of the tissue resulting from swelling. Examples of possible morphological changes which might cause loss of potassium transport include disruption of the normal intimate relationship between mitochondria and the apical cell membrane (Anderson & Harvey, 1966). Also, cellular and goblet lumen swelling might result in disarrangement of goblet cell matrix, an event which has been correlated with loss of potassium transport (Schultz & Jungreis, 1977).

The sodium–potassium exchange of most animal cells has been held to be a mechanism of volume as well as ionic regulation (Tosteson, 1964), although other mechanisms may play also a part in cell volume regulation (MacKnight & Leaf, 1977). Lepidopteran cells do not use sodium potassium exchange as a means of intracellular volume regulation since lepidopteran blood is low in sodium and most tissues do not possess sodium–potassium ATPase (Jungreis *et al.*, 1973; Vaughan & Jungreis, 1976). The potassium concentration of midgut cells is always much higher than that of the bathing solution (Zerahn, 1977), so the cells must concentrate potassium. However it appears from the present studies that volume regulation in midgut cells is dependent on quantities of non-penetrating extracellular solute, and, in the lack of such solute, increases in external potassium actually lead to an inhibition of transepithelial potassium transport (Fig. 5). This inhibition at higher potassium concentrations might stem from the fact that potassium is itself a penetrating solute as well as a transported ion.

The dependence of potassium transport on a relatively high concentration of external disaccharide is consistent with the high amino acid and trehalose concentration of lepidopteran blood (Florkin & Jeuniaux, 1974; Wyatt, 1967). It should be noted that the composition of diet might also be an important influence on the rate of potassium transport of the midgut *in vivo*, since the lumen side is especially sensitive to the effects of penetrating solute (section 2, Results), and the concentration of small dietary organic solutes at the lumen surface might be disproportionately high as a result of the filtering effect of the overlying peritrophic membrane (Zhuzhikov, 1964).

These studies were supported by an NIH postdoctoral traineeship (GM 01276-13) and by funds provided by the Washington State University Research and Arts Committee.

Note in Proof. Recent radiolabel studies (Abramcheck, F. J., Blankemeyer, J. T., Harvey, W. R. The size of the extracellular space in the isolated midgut of *Manduca sexta*, *J. Biol. Phys.* in press) have shown that mannitol equilibrates with all of the water space of the isolated midgut while sucrose equilibrates with a smaller, constant fraction of the tissue water attributable to extracellular space; this finding is in accord with the present studies.

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