J. exp. Biol. 108, 273–291 (1984)
Printed in Great Britain © The Company of Biologists Limited 1984

ACTIVE POTASSIUM ION TRANSPORT ACROSS THE CATERPILLAR MIDGUT

I. TISSUE ELECTRICAL PROPERTIES AND POTASSIUM ION TRANSPORT INHIBITION

BY M. V. THOMAS AND T. E. MAY

Shell Research Limited, Sittingbourne Research Centre, Sittingbourne, Kent, ME9 8AG, U.K.

Accepted 16 June 1983

SUMMARY

Active potassium ion transport by isolated midguts of *Spodoptera littoralis* and *Manduca sexta* caterpillars has been studied by electrical means. In contrast to previous studies, the electrical properties of the midguts remained essentially constant for several hours; this improvement probably results from use of an experimental saline that more closely resembles caterpillar haemolymph. The active transport could be abolished by anoxia and by a number of chemical agents, of which trimethyl tin chloride (effective at 10^{-9} M) was the most potent. Some of these substances, including trimethyl tin chloride, may have been acting directly on the potassium ion transport system. The results of varying the ionic composition of the saline suggest that potassium is the only cation that can be transported at a *significant* rate. However, the rate of potassium ion transport is increased by the simultaneous presence of other inorganic cations.

Experiments to determine the 'reversal potential' for the active transport pathway, by varying the potassium ion concentration, suggested that this parameter was not a constant, and thus the active transport system could not be modelled by a simple equivalent electrical circuit, although the midgut epithelium is not unique in this respect. Therefore, the tissue electrical properties could not readily be correlated with the energetics of the potassium transport process, but the results are nevertheless consistent with a potassium ion: ATP ratio of greater than one, if ATP is indeed the primary energy source.

INTRODUCTION

The active transport of inorganic ions is a general feature of living cells. If the cells are in contact to form a continuous epithelium, then net transport of ions across the epithelium will result if the active transport systems are asymmetrically distributed between the cell membranes that constitute its two surfaces. In the extreme case, the active transport system for a particular ion may be present only on one surface, with the other surface being sufficiently permeable to allow passive (i.e. energy-independent) transfer of the ion at a rate sufficient to match that of the active system.

Key words: Potassium transport, transepithelial ion transport, caterpillar midgut.

A number of 'classical' models of transepithelial ion transport such as Koefoed-Johnson & Ussing's (1958) model of Na^+ transport across the frog skin, are of this form.

The midgut of lepidopteran larvae has proved to be a very interesting model ion-transporting system in several respects. It was shown by Harvey & Nedergaard (Harvey & Nedergaard, 1963, 1964; Nedergaard & Harvey, 1968) that midguts isolated from the silkworm *Hyalophora cecropia* generated potentials of around 80 mV (lumen-side positive), and were able to transport potassium from the haemolymph (basal) to the lumen (apical) side of the tissue at a very high rate. They found a close agreement between the potassium flux and the current generated by the tissue when it was short-circuited, suggesting that the active movement of potassium was not coupled to that of any other ion. The potassium ion transport system is also insensitive to ouabain (Jungreis & Vaughan, 1977) and these findings make it unlikely that the ATP-dependent sodium/potassium pump is involved. Ultrastructural studies by Anderson & Harvey (1966) showed the epithelium to be a single cell layer, but composed of two cell types which they termed columnar cells and goblet cells, the former being approximately twice as numerous. They proposed that the potassium transport system was located on the apical membranes of the goblet cells, as these were closely associated with mitochondria. Support for the apical location of the pump was given by microelectrode experiments (Wood, Ferrand & Harvey, 1969; Blankemeyer & Harvey, 1978), which are discussed in the accompanying paper (Thomas & May, 1983). It is not clear whether an identical pump exists in vertebrate species (see Keynes, 1969), but it does occur in other insect tissues such as the salivary glands of *Calliphora* (Oschman & Berridge, 1970) and the Malpighian tubules of *Rhodnius*, *Carausius* and *Calliphora* where it can transport sodium ions as well (Maddrell, 1977; see also Harvey, 1980).

Studies of midgut potassium transport in caterpillars other than *H. cecropia* have yielded essentially similar results. Other species studied included *Antherea pernyi* (Wood, 1972), *Manduca sexta* (Blankemeyer & Harvey, 1978; Moffett, 1979, 1980) and *Philosamia cynthia*, *Macrothylatia rubi* and *Bombyx mori* (Giordana & Sacchi, 1977). These preparations all generate transepithelial potentials of around 60–80 mV and short-circuit currents, I_{sc}, of a few hundred $\mu A\,cm^{-2}$.

Unfortunately, all previous experimental studies have been complicated by the progressive and irreversible decline in these parameters *in vitro*. Ultrastructural studies by Schultz & Jungreis (1977) have suggested that the decline is a result of cell deterioration and death, which these authors attributed to inadequacies in the salines used for *in vitro* experiments.

Another factor that has impeded progress in understanding the potassium transport system has been the lack of a specific inhibitor analogous to ouabain. Such an inhibitor would be useful if not essential for characterization of the system at the biochemical level, a process which has scarcely begun. It is not even certain that the potassium ion transport system is ATP-dependent, although a potassium-stimulated ATPase activity in *M. sexta* midguts has been briefly reported in an abstract (Wolfersberger, 1979). Measurements of ATP content and the redox state of mitochondria in *M. sexta* midguts during anoxia showed that the decline in I_{sc} lagged behind the reduction of the respiratory enzymes, so there must be an intermediate link between respiration

and potassium transport. Furthermore, ATP levels were found to fall sufficiently slowly for this substance to perform such a function (Mandel, Riddle & Storey, 1980). Thus it appears to be at least a reasonable working hypothesis that the potassium transport system is powered by ATP hydrolysis, although there is still considerable uncertainty.

Since this transport system is of relatively great importance in insects, a better understanding of it, and of the substances that can interfere with it, is of correspondingly great interest. This paper describes the results of such a study on *Spodoptera littoralis* caterpillars. Particular attention was paid to maintaining the viability of the midgut tissue *in vitro*, so that the effects of substances that may modulate the rate of potassium transport could be more reliably assessed. To demonstrate that our success in this direction was a result of the experimental procedures employed rather than our choice of preparation, a few experiments were also performed on *M. sexta* midguts.

MATERIALS AND METHODS

Experimental material

Most experiments were performed on caterpillars of the Egyptian armyworm, *Spodoptera littoralis*, as this important pest species is routinely used in insecticide screening tests at Sittingbourne Research Centre. Partly for comparative purposes, some experiments were also performed on the tobacco hornworm, *Manduca sexta*, which also has the advantage of being larger in size. All caterpillars were leaf-reared (spinach or cabbage for *S. littoralis* and tobacco for *M. sexta*).

Mature, but still actively feeding, final instar larvae were used in all experiments. The caterpillars were chilled on crushed ice for at least 1 h prior to dissection, which was carried out either on ice or on a cooled microscope stage. The midgut was excised and a portion of its anterior region was mounted as a flat sheet between two concentric plastic washers having outer diameters of 7 mm and 4 mm. This was achieved by sliding the smaller washer sideways into the gut lumen, and then pushing the larger washer over it. The rest of the gut and one of the two surfaces were then dissected away, leaving a single tissue layer trapped between the washers as shown in Fig. 1.

The area of tissue thus exposed to the saline in the experimental chamber was $0{\cdot}03\,cm^2$. This was mounted into the central dividing wall of a two-compartment chamber using silicone grease, again as shown in Fig. 1.

Each compartment was perfused with saline at $0{\cdot}5\,ml\,min^{-1}$, and oxygen bubbled into each compartment through fine glass capillary tubing. This served both to oxygenate the midgut tissue and to circulate the saline in the two chambers.

Electrical recording

For voltage-clamping the transepithelial potential to 0 mV (for short-circuit current, I_{sc}, measurements) or to other potentials, a three-electrode system was used, based on the one described by Wood & Moreton (1978). Under conditions of relatively high current flow, the transepithelial potential may differ significantly from the potential recorded between the two measurement electrodes (VA and VB), as a consequence of the voltage drop in the bath due to the resistance of the saline. The

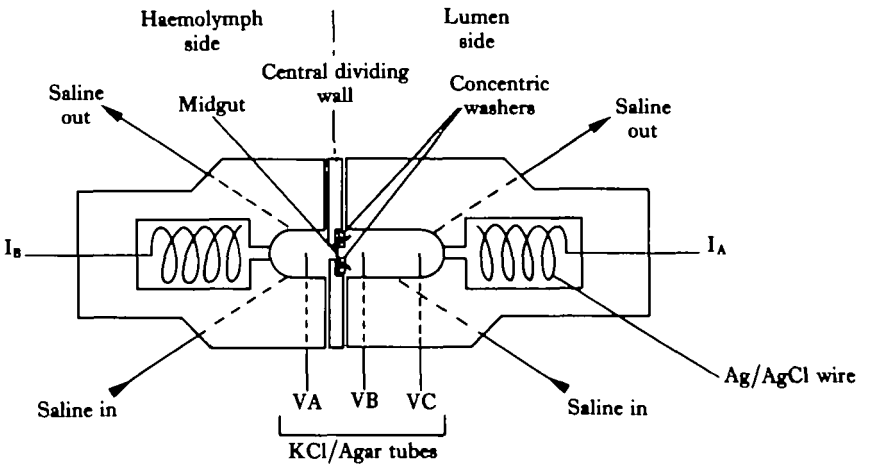

Fig. 1. Design of the experimental chamber.

same error voltage appeared between VB and VC, as the spacing between these electrodes (1 cm) was identical to that between VA and VB. It was used to correct for the effect in the same way as described by Wood & Moreton, using the circuit shown in block form in Fig. 2. The voltage-clamp potential was determined by the potential applied to the 'set V' input, and the current was measured by a virtual ground circuit connected to the electrode I_B. The tissue was mounted in the bath so that its haemolymph side faced VA, and the potential was measured relative to this side, in accordance with the convention adopted by other workers.

The voltage electrodes were silver/silver chloride pellets mounted in Perspex holders, made by W. P. Instruments and obtained from Clark Electromedical Supplies, Pangbourne, Reading, U.K. The pellets were immersed in 3 M-KCl and connections to the bath were made with agar bridges which also contained 3 M-KCl. The current-passing electrodes consisted of about 15 cm of chloride-coated silver wire (22 AWG) immersed in 3 M-KCl and connected to the bath by 3 M-KCl/agar bridges.

Haemolymph analysis and saline composition

A problem that has complicated many previous experiments on ion transport across caterpillar midguts is the progressive decline in the rate of transport normally observed after their isolation.

In our experiments we were particularly anxious to minimize this decline, as in many of them we wished to test the effects of various substances on the rate of potassium ion transport. Accordingly, considerable effort was expended on devising a saline whose composition closely resembled that of the haemolymph. To this end, the composition of the haemolymph and of the gut contents of final instar *S. littoralis* larvae was investigated, and the results are shown in Table 1. Determinations of sodium and potassium levels were made by flame photometry, those of Ca^{2+} and

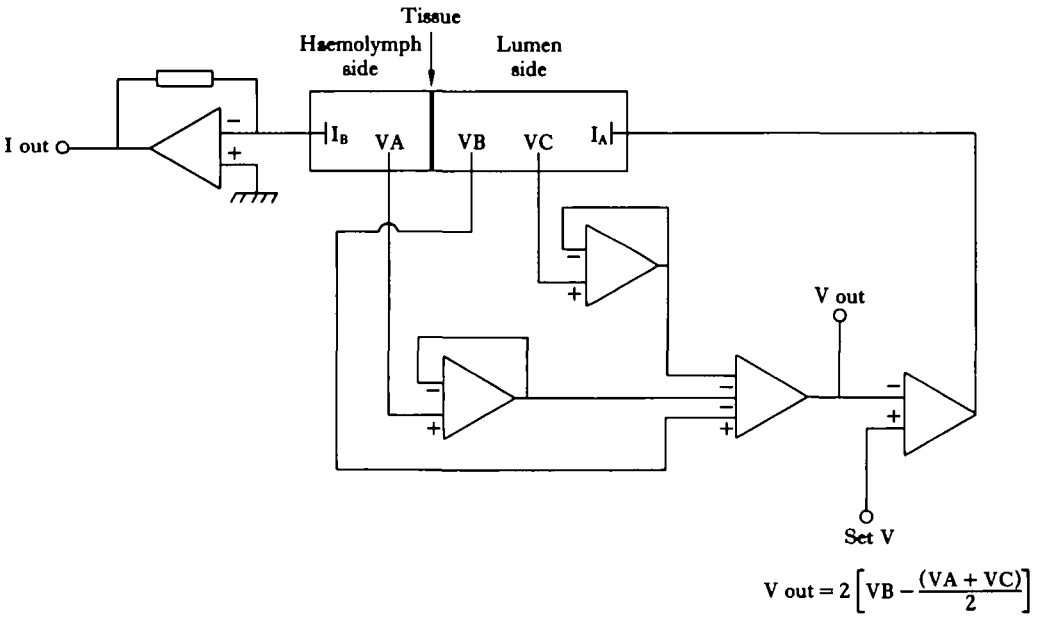

Fig. 2. Block diagram of the electrical recording system.

Mg^{2+} by atomic absorption spectrophotometry (Perkin Elmer 306), those of Cl^- with an EEL coulimetric chloride meter and those of citrate by u.v. spectrometry. The osmolarity of the haemolymph was determined using a Halbmikro osmometer, and the pH was determined with a microcapillary pH assembly.

It is clear from Table 1 that chloride is a relatively minor anion and that the major anions must be organic ones, such as citrate. The potassium and citrate levels could be approximated reasonably well by use of about 14 mM-K_3 citrate, and the Mg^{2+} and (unidentified) organic anion concentrations were approximated by 45 mM Mg gluconate. Other organic anions were represented by use of the anionic pH buffer morpholinopropane sulphonic acid (MOPS). The full composition of the saline was: 20 mM-NaOH, 13·8 mM-K_3 citrate, 11 mM-$CaCl_2$, 45 mM-Mg gluconate, 40 mM-MOPS,

Table 1. *Analysis of haemolymph and gut lumen contents from final instar* Spodoptera littoralis *caterpillars*

Parameter	Haemolymph Mean ± S.E.	No. of larvae tested	Gut lumen Mean ± S.E.	No. of larvae tested
pH	6·54 ± 0·3	10	9·1 ± 0·4	9
Osmolarity (mosmol l^{-1})	284 ± 32	12	–	0
Na^+ (mM)	20 ± 8·0	24	1·0	5
K^+ (mM)	41·5 ± 7·5	24	230 ± 20	23
Cl^- (mM)	15 ± 9·0	24	0·8 ± 0·2	5
Citrate (mM)	14·5 ± 3·1	8	4·9 ± 1·0	2
Ca^{2+} (mM)	11 ± 5·0	24	2·0 ± 0·8	5
Mg^{2+} (mM)	4·5 ± 5·0	24	0·9 ± 0·6	5

60 mM-sucrose. This saline was adjusted to pH 7·0 by addition of HCl as necessary, and its osmolarity was measured as 280 mosmol l^{-1}. Although specifically intended for *S. littoralis*, it was also used in the *M. sexta* experiments to allow the results to be compared.

Most other studies of caterpillar midgut potassium ion transport have been performed using the '32K-S' saline devised by Harvey & Nedergaard (1964). For comparison, its composition is 30 mM-KCl, 2 mM-$KHCO_3$, 5 mM-$CaCl_2$, 5 mM-$MgCl_2$, 5 mM-TrisHCl (pH 8·0) and 166 mM-sucrose.

In all experiments reported here, the ionic compositions of the two bathing media were kept identical, to ensure correct operation of the three-electrode voltage-clamp system. When the ionic composition was varied, the sucrose concentration was adjusted to maintain the same osmolarity.

RESULTS

Transepithelial potential and short-circuit current

With the saline used in these experiments, *S. littoralis* midguts generated transepithelial potentials and short-circuit currents that could be stable within about 10 % over periods of several hours. In some preparations a rapid decay in both parameters was observed soon after transfer of the epithelium to the experimental chamber, in which case the tissue was discarded. In the majority of cases, however, there was a steady *increase* in both parameters over the first few minutes, and they then typically remained stable within about 10 % over the next several hours. Mean values (±S.D.) observed during this time were 76·5 ± 12·5 mV transepithelial potential and 31·7 ± 12·5 μA short-circuit current (137 experiments).

Similar results were obtained with *M. sexta*, using the same saline, where mean values (±S.D. in five experiments) of 86·4 ± 12·2 mV and 29·7 ± 5·0 μA were obtained. These values were also stable for several hours. Since the nominal tissue area in these experiments was 0·03 cm^2, the mean short-circuit current for *S. littoralis* is 1057 μA cm^{-2} and the mean tissue resistance is 72·4 Ωcm^2. The corresponding values for *M. sexta* are 990 μA cm^{-2} and 87·3 Ωcm^2.

Current-voltage relations

Measurements of I_{sc} are a very useful quantitative method of studying the rate of K^+ transport. The method can be extended to investigate the current when the tissue is held at any potential, and such measurements can provide additional information about the potassium ion transport system.

Current-voltage (I-V) relations can be measured either 'instantaneously' using voltage pulses or under steady-state conditions; the two methods will not necessarily give identical results, since the current may take several seconds to reach a new steady value following a change in potential. This phenomenon was studied for *M. sexta* midguts by Moffett (1980), where the results from the two methods were not greatly different, although the I-V curves generated by the pulse method were somewhat more linear. In the present experiments (on both *S. littoralis* and *M. sexta*) the differences were found to be no greater than those found by Moffett on *M. sexta*, and

the steady-state method was used routinely as it was the more convenient. Currents were normally measured 30 s after a voltage change, or sometimes after a shorter period if this was sufficient for complete stabilization.

Fig. 3 shows a typical experiment, and it also illustrates the effects of anoxia. The I-V relation was very linear over the range of potentials studied (0–100 mV in this experiment) and it changed very little over a 2-h period. When nitrogen rather than oxygen was bubbled into both compartments of the chamber, the short-circuit current was abolished and there was also a reduction in the slope of the I-V relation. Reversibility of the effect was essentially complete, as is demonstrated by the I-V relation obtained 1 h later in normal saline.

In this experiment, I_{sc} declined by less than 25 % over a 3-h period, whereas other workers have reported a much greater time dependence. For example, Wood & Moreton (1978) observed a decline of approximately 70 % in the I_{sc} generated by *H. cecropia* midguts over the same period (their Fig. 5). Even so, the decline observed in Fig. 3 was relatively large (e.g. see Fig. 5 below). Thus, in contrast to other studies (e.g. Nedergaard & Harvey, 1968; Moffett, 1979) we did not need to compensate for

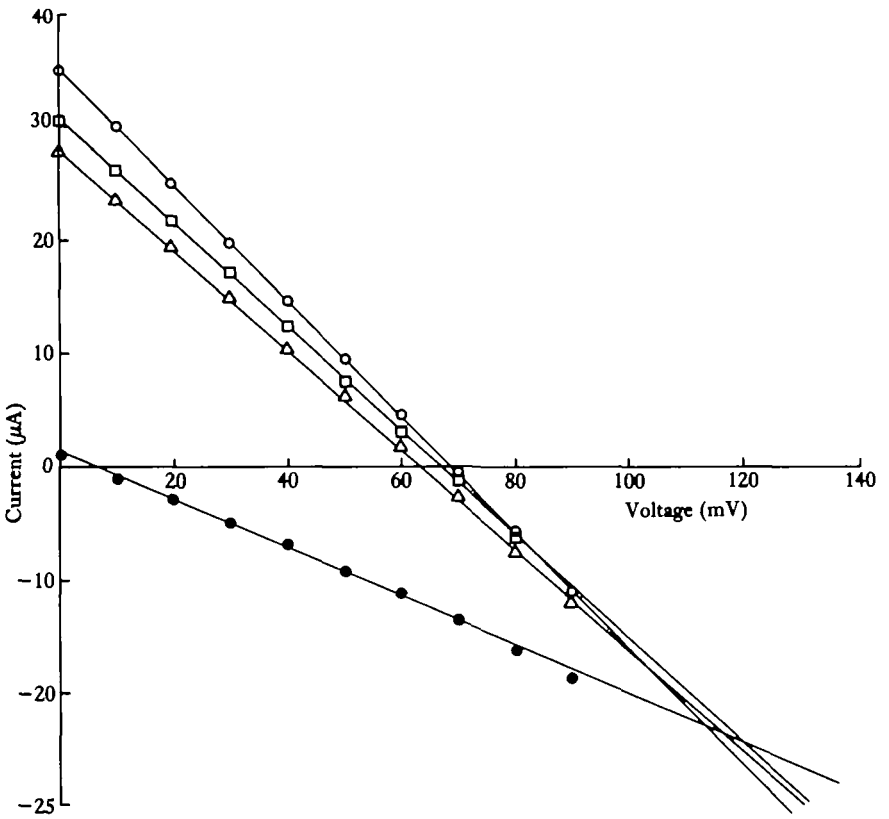

Fig. 3. Current-voltage relations of a *Spodoptera littoralis* midgut, to show the effects of anoxia. Open circles show the relation immediately after transfer of the tissue to the experimental chamber, open squares show the relation 2 h later, just prior to anoxia, solid circles show the relation during anoxia, and open triangles show the relation 1 h after anoxia.

it when investigating the effects of various procedures on the electrical properties of isolated midguts.

Extrapolation of the I-V relations in the presence and absence of oxygen predicts an intercept at approximately +120 mV. If (as seems reasonable) anoxia is presumed to abolish completely the active transport of potassium, the intercept potential should correspond to the potential that must be applied to abolish the active transport of potassium under normal conditions, i.e. to the 'reversal potential', E_K, of the potassium transport system (cf. Yonath & Civan, 1971). This concept will be discussed further below.

Effects of various substances on the rate of potassium ion transport

We were particularly interested in finding substances that specifically inhibited the potassium ion transport and to this end a wide variety of substances were tested. They were applied to either or to both sides of the tissue, and it was always found that their effects (if any) were greater when applied on the haemolymph side than on the lumen side of the epithelium. This finding is of considerable interest in that the potassium transport system is believed to be located on the luminal surface of the epithelium (Anderson & Harvey, 1966; Blankemeyer & Harvey, 1978; Thomas & May, 1983) so the opposite effect might have been expected. It will be discussed in more detail subsequently, but in consequence of it, all the results given in this section are for application to the haemolymph side unless stated otherwise.

The substances tested can be divided into four major classes, (i) those known to affect active and passive movements of ions through membranes; (ii) those known to inhibit specific aspects of metabolism; (iii) other substances, known to be toxic to caterpillars, but of unknown mode of action and (iv) putative transmitter substances that may perhaps modulate the rate of potassium transport. Most substances were tested at an initial concentration of 10^{-3} M under short-circuit conditions.

In group (i) very few substances showed activity. Greatest activity was shown by 9-aminoacridine (Fig. 4), which caused a 75 % reduction in I_{sc} at 10^{-3} M. This substance inhibits both sodium and potassium channels in excitable tissues (Yeh, 1979). The related substances acriflavine and thionine were also partially effective, giving respective reductions of 70 % and 55 %. All these effects were reversible on return to normal saline. The other major substances known to inhibit potassium movement, namely tetraethylammonium ions and a variety of aminopyridines (4-aminopyridine 3,4-diaminopyridine and 2,6-diaminopyridine) were all ineffective at 10^{-3} M. The only other substance in this series to show appreciable activity was ethacrynic acid (Fig. 4). This substance (which reduces the cation permeability of renal tubules) caused a 35 % inhibition of I_{sc} at 10^{-3} M, with a total block at 10^{-2} M. The ineffectiveness of cardiac glycosides such as ouabain is already well documented for other caterpillar midgut preparations (Jungreis & Vaughan, 1977) and we found ouabain to be ineffective at 10^{-4} M, although the experimental conditions (room temperature in the presence of a high potassium concentration) were admittedly somewhat unfavourable.

The cation ionophore antibiotics, gramicidin and valinomycin, had no effect at 10^{-5} M. Their high cost and poor solubility precluded tests at higher concentrations, but a crown ether ionophore (18-crown-6) caused only a 20 % inhibition of I_{sc} when tested at 10^{-2} M.

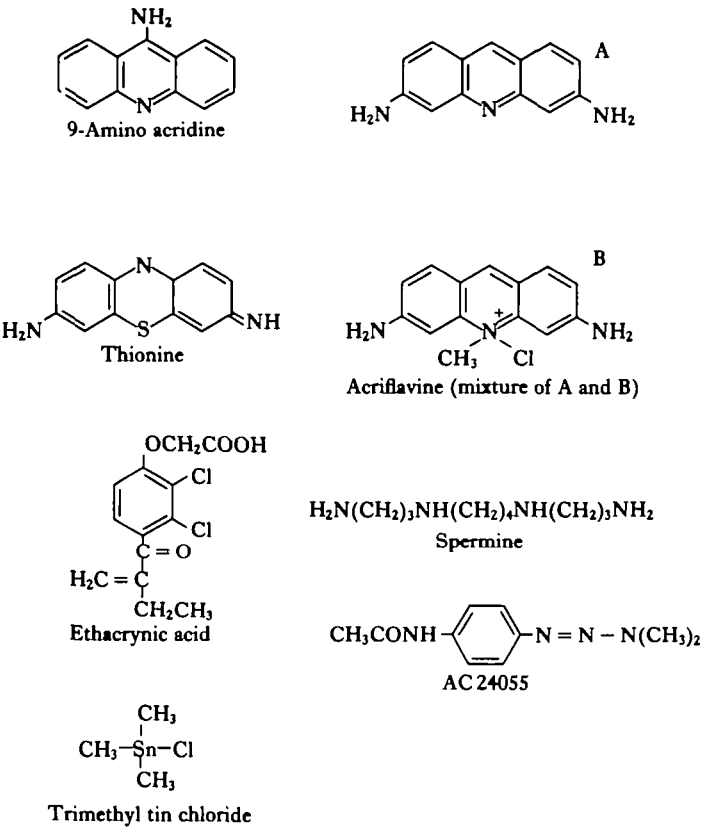

Fig. 4. Structures of some of the substances that inhibited K^+ transport.

The polyamine spermine (Fig. 4) caused a 40% reduction at 10^{-3} M. This substance has been found to be a non-competitive glutamate antagonist in insect larval muscle, its action apparently being to block cationic channels (Robinson, 1980).

As expected, a fairly large number of substances in group (ii) (metabolic inhibitors) were effective. These included cyanide (complete block at 10^{-3} M), oligomycin (complete block at 5×10^{-6} M), antimycin A (20% inhibition at 10^{-6} M), atractyloside (25% inhibition at 10^{-3} M), cycloheximide (20% inhibition at 10^{-3} M) and sodium orthovanadate (15% inhibition at 10^{-3} M; 70% inhibition at 10^{-2} M). Compounds inactive at 10^{-3} M, and for which some effect might have been expected, included rotenone and sodium arsenate. The plant lectin concanavalin A was ineffective at 10^{-4} M.

Few compounds in group (iii) were active, but two were of particular interest in that they *may* have been relatively specific inhibitors of the potassium ion transport process. These were the American cyanamid insecticide AC 24055 (Fig. 4) which caused an irreversible block at 10^{-4} M, and trimethyl tin chloride (Fig. 4), which caused a total and incompletely reversible block at 10^{-9} M. The effects of trimethyl tin were particularly interesting in two respects. First, this substance was very much less effective when applied on the lumen-side of the tissue, where a concentration of

10^{-3} M was required for a comparable effect. For most other compounds the difference in potency on the two sides was very much less, ratios of 10- to 100-fold being typical. Second, the tin had to be as a methyl derivative to show activity. For example, tripropyl tin chloride was completely ineffective on either side of the tissue at 10^{-3} M. Interpretation of the effects of trimethyl tin is difficult, but a direct effect on the potassium ion transport system is a distinct possibility (see Discussion).

The potassium transport system may well be under some form of hormonal control and the compounds in group (iv) were tested to investigate this possibility. The irreversible loss of potassium ion transport capacity during larval–pupal transformation has already been reported (Haskell, Harvey & Clark, 1968) and we were interested in the possibility that the potassium ion transport may be reversibly modulated during the larval stage. However, none of the substances examined had any effect at 10^{-3} M. These were 5-hydroxytryptamine, octopamine, dopamine, tyramine, noradrenaline, proctolin, acetylcholine and glutamic, aspartic and γ-aminobutyric acids. These negative results, although disappointing, do not, of course, rule out the possibility that other substances may modulate the rate of potassium transport, and a more rigorous investigation could well prove worthwhile.

Effects of saline composition

Several reports have suggested that caterpillar midguts can actively transport ions other than potassium. For *H. cecropia* midguts these include rubidium (Nedergaard & Harvey, 1968), caesium (Zerahn, 1970) and – in the absence of potassium – sodium and lithium as well (Harvey & Zerahn, 1971). These ions are all transported from the haemolymph to the lumen, but active transport of magnesium and calcium ions in the reverse direction has also been demonstrated (Wood, Jungreis & Harvey, 1975; Wood & Harvey, 1976).

In view of these reports, we were interested in investigating to what extent ions other than potassium could be actively transported by the *S. littoralis* midgut preparation. The procedure used in these experiments was to compare the I_{sc} obtained in various salines with that obtained in a saline containing potassium as the only cation, as it was found to provide the best illustration of the interactions which we observed. The compositions of the two bathing media were always changed together to allow correct operation of the three-electrode voltage-clamp system, and to facilitate interpretation of the results.

The results are well summarized by the experiment shown in Fig. 5. The reference saline contained 40 mM-potassium (as KOH titrated to pH 7 with MOPS) with its osmolarity balanced to that of the normal saline by addition of sucrose. The I_{sc} in this saline was 12·5 μA, and it increased to 20 μA when 20 mM-sodium citrate was added (pH and osmolarity were adjusted to compensate). When potassium ions were omitted from the saline (sodium now as NaOH, titrated with MOPS), the I_{sc} was reduced to 4 μA. On the two subsequent occasions when potassium ions were omitted, the I_{sc} was again reduced to the same value, even though on one occasion the saline contained Ca^{2+} (as 11 mM-$CaCl_2$) and on the other, Mg^{2+} (as 45 mM-Mg gluconate).

Thus potassium ions must be present, irrespective of other ions, for the tissue to generate an appreciable I_{sc}. However, Fig. 5 also shows that in the presence of potassium ions, other ions greatly increase the I_{sc}. When Na^+ (20 mM-Na citrate) was

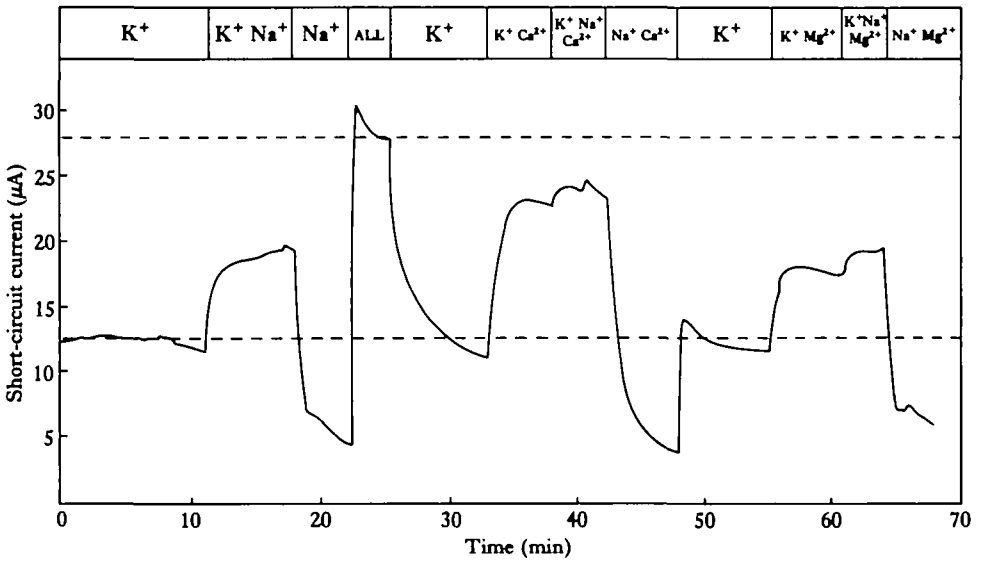

Fig. 5. Effects of saline composition on the short-circuit current of a *Spodoptera littoralis* midgut. The upper dotted line represents the short-circuit current with all ions present, and the lower dotted line represents the short-circuit current when K^+ is the only cation.

added to the saline, the I_{sc} increased to 20 μA, whereas Ca^{2+} (as 11 mM-$CaCl_2$) and Mg^{2+} (45 mM-Mg gluconate) increased it to 22 μA and 17 μA respectively. In the normal saline, which contained all these ions, the I_{sc} was 27·5 μA.

This experiment clearly shows that the I_{sc} in the presence of potassium ions (and by implication the rate of potassium transport) is strongly dependent on the saline composition, and that the interaction is a complex one in that the effects of the individual components are less than additive. They also suggest that, under these experimental conditions, ions other than potassium are not transported at a significant rate in the absence of potassium.

Interpretation of these results is complicated by the problem that both the cationic and the anionic composition of the saline is being varied, although other experiments have suggested that replacement of the organic anions by chloride does not substantially affect the I_{sc} in the *short term* (see Discussion). Nevertheless, this method of study is not very suitable for investigating the nature of the effect.

A more informative method is to investigate the potassium-dependence of the I_{sc} in the presence and in the absence of other ions. The method can be extended to measurement of the transepithelial current at a variety of potentials, and the results of two experiments are shown in Figs 6 and 7. In the experiment shown in Fig. 6, the potassium-free saline contained only sucrose and 50 mM-Tris base, titrated to pH 7·0 with HCl. (Tris was used in place of MOPS so that the saline contained no inorganic cations). Potassium was added as the citrate salt, with the sucrose concentration reduced accordingly to maintain a constant 280 mosmol l^{-1}.

As the potassium concentration was increased, there was an increase in both I_{sc} (top curve) and tissue conductance (as judged by the increasing separation between the curves obtained at the various different holding potentials).

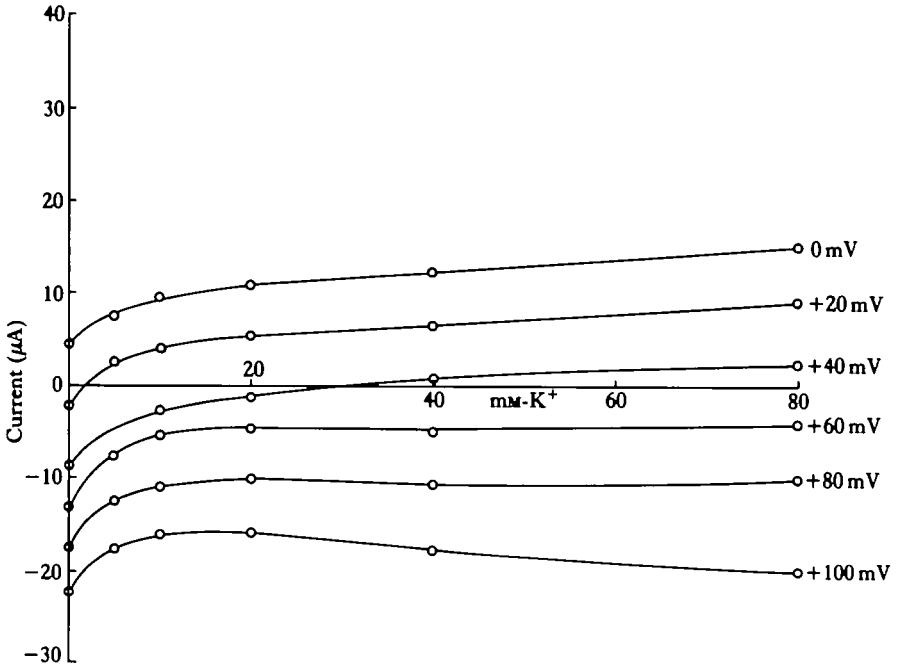

Fig. 6. Potassium-dependence of the current flow across a *Spodoptera littoralis* midgut at various potentials, in the absence of other inorganic cations (K^+ replaced by sucrose).

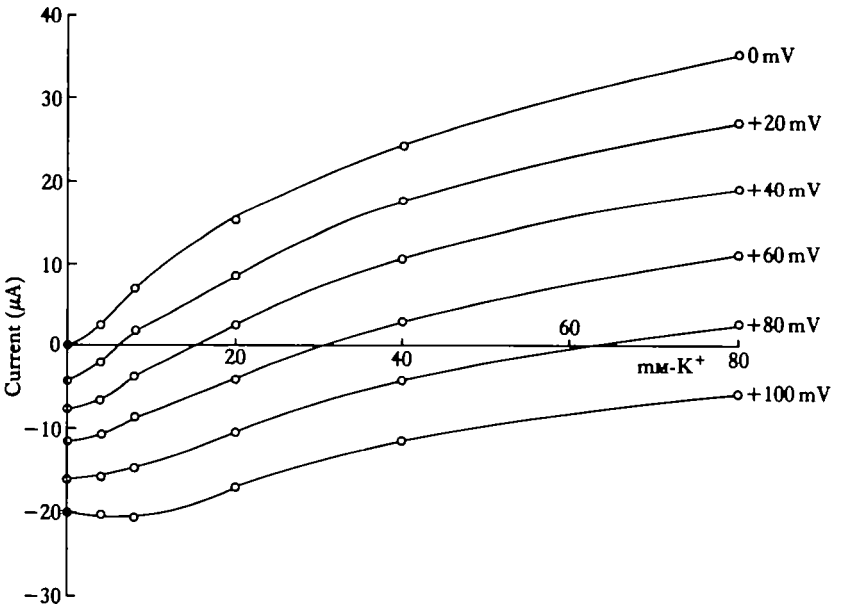

Fig. 7. Potassium-dependence of the current flow across a *Spodoptera littoralis* midgut at various potentials, with all other inorganic cations present (K^+ replaced by sucrose).

The I_{sc} was much less potassium-dependent for concentrations above 20 mM, although it was still increasing slightly up to 80 mM. If the I_{sc} at 80 mM-potassium is taken as a true maximum, then I_{sc} max = 15 μA and the potassium concentration for half-maximal I_{sc} = 5 mM.

Fig. 7 shows the result of a corresponding experiment in which all components of the normal saline were present. For direct comparison with Fig. 6, however, Tris HCl rather than MOPS was again used as the buffer. As before, potassium was added as citrate, with the sucrose concentration adjusted to compensate.

As in Fig. 6, there was no I_{sc} in the absence of potassium, which suggests that only potassium can be transported under these conditions. In this case, however, the I_{sc} was potassium-dependent over the entire concentration range, and I_{sc} max had to be estimated by extrapolation. A double-reciprocal plot of I_{sc} against potassium concentration (not shown) fitted the data points for concentrations $\geq$8 mM very well, although the 4 mM point deviated significantly from this line and was not included in the analysis. The best fit line gave I_{sc} max = 64 μA and potassium concentration for half maximal I_{sc} = 65 mM.

Similar values were obtained in three other experiments where potassium was replaced by sodium, so that the anionic composition of the saline remained constant. The results from a single experiment on *M. sexta* were within the same range. The results of all these experiments are shown in Table 2. With the exception of the first result (where potassium was the only inorganic cation), the differences between the individual experiments are clearly not significant.

The experiment shown in Fig. 7 has been replotted as a series of I-V relations in Fig. 8, to illustrate the potassium-dependence of the tissue conductance more clearly. The conductance, represented by the slope of the I-V relation, increased with the potassium concentration, approaching a maximum value at the highest concentrations.

Since the conductance is a possible alternative measure of the rate of potassium transport (see Discussion) it is expected to show a similar potassium-dependence to the I_{sc}. An analogous double-reciprocal analysis suggested that the potassium concentration for half-maximal conductance increase (from that in potassium-free saline) was 51 mM, which is in reasonable agreement with the 65 mM calculated for half-maximal I_{sc}.

It will be noted, however, that the I-V relations obtained for the various potassium

Table 2. *Effects of saline composition on short-circuit currents of individual caterpillar midguts*

Species	Inorganic cations present	K^+ replaced by	$[K^+]$ for half-max I_{sc}	I_{sc} max
S. littoralis	K^+ only	Sucrose	5 mM	15 μA
S. littoralis	All	Sucrose	65 mM	64 μA
S. littoralis	All	Na^+	60 mM	66 μA
S. littoralis	All	Na^+	37 mM	74 μA
S. littoralis	All	Na^+	29 mM	68 μA
M. sexta	All	Na^+	44 mM	65 μA

The first results are shown in detail in Figs 6–8.

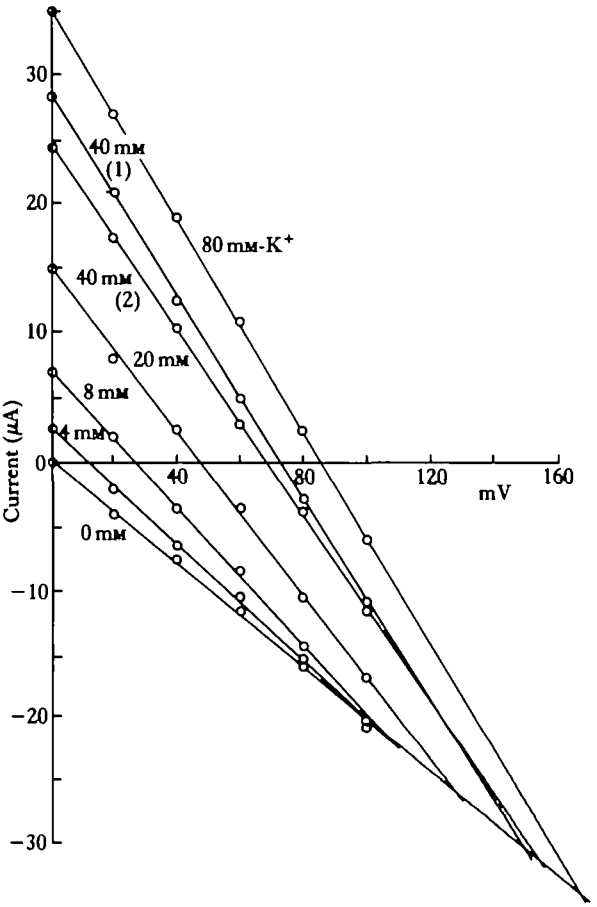

Fig. 8. The data of Fig. 7, replotted in the form of a series of current-voltage relations as a function of K^+ concentration.

concentrations intersect the I-V relation obtained in zero potassium at significantly different potentials. The significance of this very interesting finding will be considered in the Discussion.

DISCUSSION

Ionic composition of caterpillar haemolymph and of experimental salines

As expected, the results of our haemolymph analysis on *S. littoralis* are qualitatively similar to those obtained in other caterpillars. Mg^{2+} is the dominant cation in the haemolymph, as found by Jungreis, Jatlow & Wyatt (1973) for *H. cecropia* and by Giordana & Sacchi (1978) for both *Philosamia cynthia* and *Bombyx mori*. The higher concentration of potassium than of sodium in the haemolymph is also in agreement with those studies. Detailed comparison of these results does suggest, however, that the ionic composition of the haemolymph varies substantially between caterpillar species. Nevertheless, it is clear that the 'standard saline', which has been used extensively since Harvey & Nedergaard's (1964) initial study, is not a good substitute for caterpillar haemolymph. In comparison with it (see Methods), our saline contains

much more Mg^{2+} and somewhat less Cl^- and sucrose, together with a significant concentration of organic anions. The pH is also 1 unit more acid.

We did not investigate *in detail* to what extent the saline was responsible for the excellent viability of our preparations *in vitro*. Other factors, such as very efficient oxygenation of the saline may also be important, as the respiration rate of caterpillar midgut tissue is relatively high (Mandel *et al.* 1980). It was observed in one experiment, however, that replacement of Mg gluconate by $MgCl_2$ caused a progressive 30% reduction in I_{sc} over a 45-min period, only a third of which was reversible on return to normal saline. Thus we suspect that the altered saline composition was the major reason for the excellent viability of our preparations. The choice of caterpillar was certainly *not* the reason, as we obtained similarly good results with *M. sexta*, whereas this preparation shows the same characteristic decline in electrical properties as is observed for *H. cecropia* when studied under 'conventional' conditions (Moffett, 1979).

Electrical properties of S. littoralis *and* M. sexta *midguts*

When due allowance is made for the differences in viability, the potassium ion transport capacity (as judged from the I_{sc}) of the *S. littoralis* midgut is similar to that of *H. cecropia*. The mean I_{sc} of 1057 μA cm^{-2} is equivalent to 39·4 μequiv cm^{-2} h^{-1}, which compares with values of 498 μA cm^{-2} and 18·6 μequiv cm^{-2} h^{-1} for *H. cecropia* measured with a similar three-electrode system (Wood & Moreton, 1978). Those values were obtained after 1 h in the 'conventional' saline and the initial values would have been nearly twice as great (Wood & Moreton, 1978, Fig. 5 and equation 13). On this basis the results are very similar.

For *M. sexta* midguts in the same saline, Moffett (1979, Fig. 1) obtained an initial I_{sc} of 90 μA, equivalent to 409 μA cm^{-2} or 15·2 μequiv cm^{-2} h^{-1}. For comparison, we obtained values of 990 μA cm^{-2} and 36·9 μequiv cm^{-2} h^{-1}, using our *S. littoralis* saline.

The mean transepithelial potentials we obtained for *S. littoralis* (76·5 mV) and *M. sexta* (86·4 mV) were not significantly different, but they are both lower than the initial values of approximately 120 mV for *H. cecropia* in 'conventional' saline (Wood & Moreton, 1978). This may be a consequence of the difference in saline composition.

Our results for *S. littoralis* yield a mean tissue resistance of 72·4 Ωcm^2. Wood & Moreton (1978) reported a mean value of 150 Ωcm^2 for *H. cecropia* after 1 h, but the initial value was probably some 30% lower (their Fig. 5) and thus rather closer to our own. Our resistance estimate for *M. sexta* midguts was 87·3 Ωcm^2, which is close to Moffett's (1979) estimate of 78·3 Ωcm^2 for open-circuited midguts of this species.

We thus conclude that the electrical properties of *S. littoralis* midguts are not significantly different from those of *M. sexta* or of *H. cecropia*.

Inhibition of potassium ion transport

Although a number of substances were found to inhibit potassium ion transport, none of them was necessarily a specific inhibitor of the potassium ion pump itself. One substance that has been the subject of considerable recent interest is *Bacillus thuringiensis* endotoxin which inhibits potassium ion transport after exposure to the alkaline pH in the midgut (Harvey & Wolfersberger, 1979; Griego, Moffett & Spence, 1979),

but it also causes lysis of cultured insect cells (Murphy, Sohi & Fast, 1976), suggesting that potassium ion transport inhibition may result indirectly from cell damage. For this reason we did not follow up those observations in our experiments.

Of the substances we tested, trimethyl tin chloride was undoubtedly of the greatest interest. Organotin compounds are known to inhibit oxidative phosphorylation in mitochondria, but trimethyl tin is a less potent inhibitor than other low molecular weight trialkyl tins (Aldridge, 1958; Sone & Hagihara, 1964). Concentrations in the micromolar range are normally required for mitochondrial inhibition, whereas trimethyl tin chloride caused complete inhibition of potassium ion transport at a much lower concentration (10^{-9} M) and yet tripropyl tin chloride was ineffective at 10^{-3} M.

Also of considerable interest was the observation that trimethyl tin chloride was very much less potent (by a factor of 10^6) when applied on the apical (lumen) side rather than on the basal (haemolymph) side of the tissue. The results thus suggest that trimethyl tin chloride may be having a relatively specific effect on the basal membrane.

The other substances that we found to be active almost all had precedents from other physiological or biochemical systems. It was of interest that a number of substances that were expected to affect cation permeability rather than potassium ion active transport had inhibitory effects. These included 9-amino-acridine, spermine and ethacrynic acid. The American cyanamid insecticide AC 24055 *might* also have been acting in this way, but we have no firm evidence on this point.

In theory, it should be possible to gain further information on the site of action of these and other substances by virtue of their effects on the tissue current-voltage relation or on intracellular microelectrode recordings. However, neither approach was pursued in detail, because of the problem in interpreting I-V relations (described above), and because of the difficulties we uncovered when attempting to identify the transporting cells in the epithelium (see Blankemeyer & Harvey, 1978) by electrical means using intracellular microelectrodes (Thomas & May, 1983).

Effects of saline composition on potassium ion transport

The effects of varying the saline composition are complex, but they allow some clear conclusions to be drawn. In particular, the very low I_{sc} values in the absence of potassium suggest that Na^+, Ca^{2+} and Mg^{2+} are not transported at appreciable rates under our experimental conditions. On the other hand, these ions and/or their counterions increase the I_{sc} in the presence of potassium, doubling it when they are all present (Fig. 5). It is clear, however, that the effects of the individual ion pairs are less than additive, so that particular increments in I_{sc} cannot be related to the presence of particular ions. These ions probably increase I_{sc} by increasing the rate of potassium transport rather than by being transported themselves. Support is given to this interpretation by the observation that the potassium-dependence of the I_{sc} is significantly affected by other ions (Figs 6 and 7).

The reason for this effect is unclear, but it does have a possible precedent in that Moffett (1979) showed that the I_{sc} of *M. sexta* midguts was reversibly reduced when small organic solutes were substituted for sucrose in the experimental saline. Our observations could be regarded as complementary, in that they show I_{sc} to increase when sucrose is partially replaced by ionic solutes.

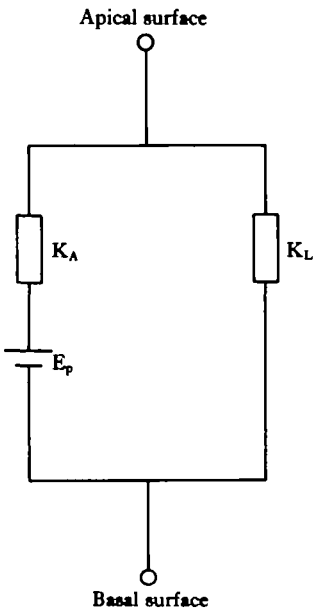

Fig. 9. An electrical equivalent circuit that is sometimes used as a model for transepithelial ion transport.

Energetics of the transport system

The electrical properties of ion-transporting epithelia can sometimes be described quite well by a relatively simple electrical circuit (e.g. Yonath & Civan, 1971), which consists of active and leakage conductance pathways (K_A and K_L) in parallel. The active conductance is in series with a battery, which represents the 'reversal potential' of the pump, E_p, as shown in Fig. 9. According to this model, E_p can be evaluated by measuring the tissue current-voltage relation both under normal conditions and when the active conductance pathway is blocked, since the two relations will intersect at this potential.

Even if the transport system can be modelled in this way, there is the problem that any procedure intended to inhibit K_A may also affect both K_L and E_p itself, so the results of such experiments should be interpreted with some caution. They may, however, be useful for comparative purposes, provided that their uncertain nature is fully taken into account.

The results presented in this paper allow E_p to be estimated by two different methods. The first is to block active transport by metabolic inhibition, such as the anoxia experiment shown in Fig. 3, which yielded an E_p value of +120 mV. The second is to remove potassium ions from the saline, as in the experiment shown in Fig. 8. This yielded a value of +150 mV when referenced with normal (40 mM potassium) saline, both at the beginning and at the end of the experiment. Estimates obtained using other potassium concentrations differed significantly, however, varying from +87 mV in 4 mM-potassium to +168 mV in 80 mM-potassium, within this same experiment.

This variation clearly cannot be due to tissue deterioration, in view of the constant

estimate for normal saline, and other explanations must be sought. A significant effect on K_L is unlikely, since K_L is relatively high, and yet under short-circuit conditions the reverse potassium flux in *H. cecropia* midguts is relatively low (Harvey & Nedergaard, 1964) suggesting that it does not contribute greatly to K_L. Explanations could perhaps be sought either in terms of non-equilibrium thermodynamics as applied to ion-transporting epithelia (Hong & Essig, 1976), or in terms of a basic inadequacy of the model itself, which is certainly a strong possibility (cf. the results of Gradmann *et al.* 1978, which showed the proton pump in the *Neurospora crassa* plasma membrane to behave as a constant current generator under some conditions, when an E_p could not be estimated at all!).

The E_p estimates given here are, however, consistent with that of +140 mV obtained by Moffett with *M. sexta*, using the potassium removal technique and referenced to 'conventional' 32 mM-potassium saline (Moffett, 1980). For comparison, E_p values reported for sodium-transporting epithelia are also of approximately this magnitude (see Moffett, 1980, for references and discussion).

If the potassium pump is powered by ATP hydrolysis, then on energetic grounds an E_p value of several hundred mV would be expected if only one potassium ion were transported per ATP molecule hydrolysed. Such a 1 : 1 ratio has been proposed for the *Neurospora* proton pump referred to above, where the E_p value was estimated to be around +400 mV (Gradmann *et al.* 1978). Although no firm conclusions can be drawn concerning the stoichiometry of the midgut potassium ion pump from the E_p estimates, the results do suggest that more than one potassium ion can be transported per ATP molecule hydrolysed.

REFERENCES

ALDRIDGE, W. N. (1958). The biochemistry of organotin compounds: trialkyl tins and oxidative phosphorylation. *Biochem. J.* **69**, 367–376.

ANDERSON, E. & HARVEY, W. R. (1966). Active transport by the Cecropia midgut. II. Fine structure of the midgut epithelium. *J. Cell Biol.* **31**, 107–134.

BLANKEMEYER, J. T. & HARVEY, W. R. (1978). Identification of active cell in potassium transporting epithelium. *J. exp. Biol.* **77**, 1–13.

GIORDANA, B. & SACCHI, F. (1977). Some ionic and electrical parameters of the intestinal epithelium in three mature larvae of lepidoptera. *Comp. Biochem. Physiol.* **56**A, 95–99.

GIORDANA, B. & SACCHI, F. (1978). Cellular ionic concentrations in the midgut of two larvae of lepidoptera *in vivo* and *in vitro*. *Comp. Biochem. Physiol.* **59**A, 17–20.

GRADMANN, D., HANSEN, U.-P., LONG, W. S., SLAYMAN, C. L. & WARNCKE, J. (1978). Current-voltage relationships for the plasma membrane and its principal electrogenic pump in *Neurospora crassa*. I. Steady state conditions. *J. Membr. Biol.* **39**, 333–367.

GRIEGO, V. M., MOFFETT, D. & SPENCE, K. D. (1979). Inhibition of active K^+ transport in the tobacco hornworm (*Manduca sexta*) midgut after ingestion of *Bacillus thuringiensis* endotoxin. *J. Insect Physiol.* **25**, 283–288.

HARVEY, W. R. (1980). Water and ions in the gut. In *Insect Biology in the Future*, (eds M. J. Locke & D. S. Smith), pp. 105–124. New York: Academic Press.

HARVEY, W. R. & NEDERGAARD, S. (1963). Alkali metal ions and the midgut potential of the Cecropia silkworm. *J. Cell Biol.* **19**, 32A.

HARVEY, W. R. & NEDERGAARD, S. (1964). Sodium-independent active transport of potassium in the isolated midgut of the Cecropia silkworm. *Proc. natl Acad. Sci., U.S.A.* **51**, 757–765.

HARVEY, W. R. & WOLFERSBERGER, M. G. (1979). Mechanism of inhibition of active potassium transport in isolated midgut of *Manduca sexta* by *Bacillus thuringiensis* endotoxin. *J. exp. Biol.* **83**, 293–304.

HARVEY, W. R. & ZERAHN, K. (1971). Active transport of sodium by the isolated midgut of *Hyalophora cecropia*. *J. exp. Biol.* **54**, 269–274.

HASKELL, J. A., HARVEY, W. R. & CLARK, R. M. (1968). Active transport by the Cecropia midgut. V. Loss of potassium transport during larval-pupal transformation. *J. exp. Biol.* **48**, 25–37.

HONG, C. O. & ESSIG, A. (1976). Effects of 2-deoxy-D-glucose, amiloride, vasopressin and ouabain on active conductance and E_{Na} in the toad bladder. *J. Membr. Biol.* **28**, 121–142.

JUNGREIS, A. M., JATLOW, P. & WYATT, G. R. (1973). Inorganic ion composition of haemolymph of the Cecropia silkmoth: changes with diet and ontogeny. *J. Insect Physiol.* **19**, 225–233.

JUNGREIS, A. M. & VAUGHAN, G. L. (1977). Insensitivity of lepidopteran tissues to ouabain: absence of ouabain binding and Na^+-K^+ ATPases in larval and adult midgut. *J. Insect Physiol.* **23**, 503–509.

KEYNES, R. D. (1969). From frog skin to sheep rumen: a survey of transport of salts and water across multicellular structures. *Q. Rev. Biophys.* **2**, 177–281.

KOEFOED-JOHNSON, V. & USSING, H. H. (1958). The nature of the frog skin potential. *Acta physiol. scand.* **42**, 298–308.

MADDRELL, S. H. P. (1977). Insect Malpighian tubules. In *Transport of Ions and Water in Animals*, (eds B. L. Gupta, R. B. Moreton, J. L. Oschman & B. J. Wall), pp. 541–569. London: Academic Press.

MANDEL, L. J., RIDDLE, T. G. & STOREY, J. M. (1980). Role of ATP in respiratory control and active transport in tobacco hornworm midgut. *Am. J. Physiol.* **238**, C10–C14.

MOFFETT, D. (1979). Bathing solution tonicity and potassium transport by the midgut of the tobacco hornworm *Manduca sexta*. *J. exp. Biol.* **78**, 213–223.

MOFFETT, D. (1980). Voltage-current relation and potassium ion transport in tobacco hornworm (*Manduca sexta*) midgut. *J. Membr. Biol.* **54**, 213–219.

MURPHY, D. W., SOHI, S. S. & FAST, P. G. (1976). *Bacillus thuringiensis* enzyme-digested delta endotoxin: effect on cultured insect cells. *Science, N.Y.* **194**, 954–956.

NEDERGAARD, S. & HARVEY, W. R. (1968). Active transport by the Cecropia midgut. IV. Specificity of the transport mechanism for potassium. *J. exp. Biol.* **48**, 13–24.

OSCHMAN, J. L. & BERRIDGE, M. J. (1970). Structural and functional aspects of salivary fluid secretion in *Calliphora*. *Tissue and Cell* **2**, 281–310.

ROBINSON, N. L. (1980). Neuromuscular blockade by polyamines at the bodywall muscles of the third instar larvae of the sheepfly, *Lucilia sericata*. In *Insect Neurobiology and Pesticide Action (Neurotox 79)*, pp. 237–238. London: Society of Chemical Industry.

SCHULTZ, T. N. & JUNGREIS, A. M. (1977). Origin of the short circuit decay profile and maintenance of the cation transport capacity of the larval lepidopteran midgut *in vitro* and *in vivo*. *Tissue and Cell* **9**, 255–272.

SONE, N. & HAGIHARA, B. (1964). Inhibitory actions of trialkyl tin compounds on oxidative phosphorylation in mitochondria. *J. Biochem., Tokyo* **56**, 151–156.

THOMAS, M. V. & MAY, T. E. (1983). Active potassium ion transport across the caterpillar midgut. II. Intracellular microelectrode studies. *J. exp. Biol.* **108**, 293–304.

WOLFERSBERGER, M. G. (1979). A potassium-modulated plasma membrane adenosine triphosphatase from the midgut of *Manduca sexta* larvae. *Fedn Proc. Fedn Am. Socs exp. Biol.* **38**, 242.

WOOD, J. L. (1972). Some aspects of active potassium transport by the midgut of the silkworm, *Antheraea pernyi*. Ph.D. thesis, University of Cambridge.

WOOD, J. L., FARRAND, P. S. & HARVEY, W. R. (1969). Active transport of potassium by the Cecropia midgut. VI. Microelectrode potential profile. *J. exp. Biol.* **50**, 169–178.

WOOD, J. L. & HARVEY, W. R. (1976). Active transport of calcium across the isolated midgut of *Hyalophora cecropia*. *J. exp. Biol.* **65**, 347–360.

WOOD, J. L., JUNGREIS, A. M. & HARVEY, W. R. (1975). Active transport of magnesium across the isolated midgut of *Hyalophora cecropia*. *J. exp. Biol.* **63**, 313–320.

WOOD, J. L. & MORETON, R. B. (1978). Refinements in the short-circuit technique and its application to active potassium transport across the Cecropia midgut. *J. exp. Biol.* **77**, 123–140.

YEH, J. Z. (1979). Dynamics of 9-aminoacridine block of sodium channels in squid axons. *J. gen. Physiol.* **73**, 1–21.

YONATH, J. & CIVAN, M. M. (1971). Determination of the driving force of the Na^+ pump in toad bladder by means of vasopressin. *J. Membr. Biol.* **5**, 366–385.

ZERAHN, K. (1970). Active transport of caesium by the isolated and short-circuited midgut of *Hyalophora cecropia*. *J. exp. Biol.* **53**, 641–649.