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MECHANISM OF INHIBITION OF ACTIVE POTASSIUM TRANSPORT IN ISOLATED MIDGUT OF *MANDUCA SEXTA* BY *BACILLUS THURINGIENSIS* ENDOTOXIN

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SUMMARY

After incubation at pH 10 or higher, *Bacillus thuringiensis* spores and endotoxin, at concentrations above 0·1 IU/ml, affected transport parameters in the isolated midgut of *Manduca sexta* larvae. (Toxic activity was lost during roughly 1 week at pH 11.) About 60% of the short-circuit current was inhibited, and the remainder was reversibly inhibited by anoxia. Electrical resistance was reduced by about 55% and oxygen uptake stimulated by about 30%. Influx of potassium from blood-side to lumen-side ('active' flux) was unaffected but flux in the reverse direction was nearly tripled. These results suggest that hydrolysis of the toxin yields an inhibitor of potassium transport, presumably a polypeptide. It is argued that inhibition is not primarily by uncoupling of oxidative phosporylation, but instead by interference with an active depression of the efflux of potassium from lumen-side to blood-side.

INTRODUCTION

Bacillus thuringiensis endotoxin is widely used as an insecticide in the control of certain plant-feeding insects. There is abundant evidence that the toxin affects potassium regulation *in vivo* (Faust & Travers, 1979) but the effect is believed to be secondary to the uncoupling of oxidative phosphorylation (Travers, Faust & Reichelderfer, 1976). Previously, the toxin has been found to have no effect on potassium transport, as measured by short-circuit current, in the isolated midgut of larvae of *Hyalophora cecropia* (J. A. Haskell, T. A. Angus & W. R. Harvey, unpublished observations made about 15 years ago) and *Manduca sexta* (D. F. Moffett, recent personal communication). It is known that the toxin is activated in the insect gut (Faust, Adams & Heimpel, 1967) and this could explain why Griego, Moffett & Spence (1979) have found inhibition of short-circuit current in midgut isolated from *M. sexta* 4 h after administration of toxin *per os*. Activation in the gut is by alkaline and enzymic hydrolysis (Faust, Hallam & Travers, 1974*a*) so in this paper we investigate whether alkalinity can promote an effect of the toxin upon transport parameters measured in the isolated midgut of *M. sexta*.

Manduca sexta is rapidly replacing *Hyalophora cecropia* in studies of active potassium transport by isolated Lepidopteran midguts because it is easier to rear in the laboratory.

The musculature of the midgut is less developed and it has a greater abundance replacement cells than the midgut of Cecropia (compared Cioffi, 1979 with Anderson & Harvey, 1966). Blankemeyer (1976) found that such transport properties as open circuit PD, short-circuit current, resistance, unidirectional fluxes and influx pool sizes are similar in midguts from the two species while Mandel and associates (1979*a*) have found similarities in oxidative metabolism of the two. With caution, then, our working assumption is that transport phenomena in the two species are similar unless proven otherwise.

MATERIALS AND METHODS

Fifth-instar larvae of *Manduca sexta* (Lepidoptera, Sphingidae), approximately 2–3 days after moulting and weighing 6–8 g, were used for these experiments. These animals were obtained as third-instar larvae from Carolina Biological Supply Co. and reared on the artificial diet supplied.

The midgut chamber (Wood and Moreton, 1978) was made of Plexiglass and held the midgut as a flat sheet over an aperture of 8 mm internal diameter, corresponding to an area of 0·5 cm^2. The bathing solution in each chamber-half was circulated by a gas lift pump usually operated by oxygen at a flow rate of 5 ml min^{-1} as measured and maintained with Brooks flow meters (model 1355-01A1Fzz40, Emerson Electric Co.).

Isolation of midgut. A larva was prepared for dissection by storing in crushed ice for 30 min, followed by chilling at −20 °C in a freezer until the tips of its abdominal appendages began to turn white with frost (3–6 min). The larva was then placed on a plastic plate which had been chilled to −20 °C. The posterior section of the larva was amputated by cutting between the third and fourth pair of abdominal appendages and the anterior section by cutting between the second and third pair of thoracic appendages. The integument was cut open carefully along the dorsal midline and was spread apart exposing the midgut which was rinsed with ice-cold bathing solution (see below). A chilled, fire-polished, Pasteur pipette was carefully pushed through the length of the midgut forcing out most of its contents and used to lift and rotate the midgut while attachments to the integument were severed. The midgut was then lifted by means of the pipette into a small pool of ice-cold bathing solution on the cold plastic plate, and opened by cutting along the pipette to form a rectangular flat sheet. After the peritrophic membrane was removed with forceps the midgut was washed free of remaining contents with ice-cold bathing solution.

The aperture-containing half of the midgut chamber was positioned horizontally and filled with enough bathing solution to form a small meniscus above the aperture. The rectangular midgut was grasped at two adjacent corners, positioned so that its thin central region (Cioffi, 1979) covered the aperture, and tied with surgical cotton. After verifying that the tissue was sealed to the chamber by demonstrating that it became protuberant and did not leak with applied hydrostatic pressure, the two chamber halves were bolted together. The chamber was filled with bathing solution while being rotated into the vertical position and the bathing solution in each chamber half was replaced (blood-side first) by 10 vols. of fresh solution, the correct level being maintained by suction lines. The transepithelial potential difference was then recorded

and the midgut was short-circuited (see below) with zero time being taken as the time of this initial short-circuiting. The average weight of the portion of the midgut exposed to bathing solutions was 22 mg.

Bathing solutions were composed of 32 mM-KCl, 1 mM-$CaCl_2$, 1 mM-$MgCl_2$, 1 mM Tris-HCl buffer and 166 mM sucrose (Harvey & Zerahan, 1972). Solutions were saturated with oxygen before use. The pH of the bathing solutions was normally 8·3 but for some experiments was elevated to 9, 10 or 11 on the lumen-side of the chamber by addition of NaOH. Wood (1972) has shown that the lumen-side but not the blood-side of the isolated midgut can tolerate such extremely alkaline conditions. All pH measurements were made with a Radiometer Model 27 pH meter.

Electrical recording. The potential (PD), short-circuit current (I_{sc}) and resistance (R_m) across the epithelium were measured in the manner of Wood & Moreton (1978). This system employed three calomel electrodes to compensate for errors due to solution resistance. Agar bridges (3% agar in normal bathing solution) were used to connect the calomel electrodes to the chamber. For some experiments PD and I_{sc} were measured with an automatic apparatus (Biologic Instruments); for others PD was measured with a Keithley 602 electrometer and I_{sc} was measured with a voltage divider and ammeter (Triplett, model 630 NA). R_m was approximated from the change in PD resulting from passage of 10 μA for 10 s first in one direction and then the other, and was expressed in Ω cm^2.

Unidirectional fluxes of potassium, from blood-side to lumen-side, $J_{K(BL)}$, and in the reverse direction, $J_{K(LB)}$, were estimated using 1 mM ^{86}Rb (New England Nuclear) to indicate K flux (Wood & Harvey, 1975, 1979). Although this can be expected to give a slight underestimate of K flux, as in *Hyalophora cecropia* (Wood & Harvey, 1979), this is of no consequence in the experiments below in which we are comparing a unidirectional flux before toxin with that after toxin. For convenience and by tradition $J_{K(BL)}$ is referred to as the 'influx' and $J_{K(LB)}$ as the 'efflux'.

I_{sc} as a measure of K transport. To determine whether I_{sc} was a good measure of K transport in *M. sexta* we compared I_{sc} and $J_{K(net)}$. At 30 min, I_{sc} averaged 290 μA (S.E. 28; $n = 6$). At this time, $J_{K(BL)}$ averaged 292 μA (S.E. 63; $n = 3$) and $J_{K(LB)}$ averaged 33 μA (S.E. 11; $n = 3$), yielding a $J_{K(net)}$ of 259 μA. If we assume that values of $J_{K(net)}$ measured using Rb as a tracer should be corrected by dividing by 0·9, as in *H. cecropia* (Wood & Harvey, 1979), then we obtain a corrected value of 288 μA for $J_{K(net)}$. Thus I_{sc} is a good estimate of $J_{K(net)}$.

Oxygen uptake was measured polarimetrically with a Beckman macroelectrode (No. 325814) and Beckman oxygen analyser (Model 160) as described for *H. cecropia* by Harvey, Haskell & Zerahn (1967). This technique may have resulted in a small overestimate of oxygen uptake in the present experiments due to diffusion of oxygen through the midgut tissue, promoted by stirring of the lumen side with nitrogen. Stirring of the lumen side with nitrogen is required in the case of *M. sexta* but not in the case of *H. cecropia*. However, any overestimate should have remained the same throughout the experimental period.

Bacillus thuringiensis *spore and endotoxin preparation* (*BT*) was obtained as a commercial insecticide (BT spray; Hopkins Agricultural Chemical Company). In addition, several key experiments have been repeated with indistinguishable results using *B. thuringiensis* HD-1 spores and endotoxin freshly prepared by H. Dulmage.

RESULTS

Inhibition of I_{sc}

The influence of alkalinity on the effect of Bt upon short-circuit current was assessed in midguts isolated from larvae matched in weight, feeding state and time after moult. Each midgut was exposed to 2 IU/ml Bt (Dulmage, 1973) on the lumen side at 30 min after the initial short-circuiting. At pH 8·3 and 9 the toxin had no effect on I_{sc}, which showed the usual decline with time (Blankemeyer, 1976), as shown in Fig. 1 for pH 8·3. At pH 10 and 11, however, the current was slightly inhibited within less than 20 min after the addition of Bt, reaching a maximum inhibition of about 70% within 40 min (Fig. 1).

Preactivation of Bt

The Bt preparation could be activated before addition to the chamber, by incubation at pH 10–11. For further experiments the routine procedure was to prepare a concentrated solution of Bt in dilute NaOH (1858 IU/ml; pH 11) and allow it to stand at room temperature for 2 h before use. Since such a preparation lost most of its activity within 1 week of storage at pH 11, a fresh concentrated alkaline Bt solution was prepared each day.

Dose–response

In 12 experiments similar to that shown in Fig. 2, Bt had no effect on I_{sc} at a concentration of 0·01 IU/ml. At a concentration of 0·1 IU/ml, I_{sc} was inhibited by a mean value of 62·4% (S.E. 15·7). Increasing the concentration by ten or one hundred times had no further effect. I_{sc} was measured at 5 min intervals. In some experiments, inhibition began within 5 min after exposure to Bt; in others within 10 min.

In seven experiments, an attempt was made to wash off the Bt with fresh normal bathing solution. In no case was recovery of the I_{sc} observed.

Residual I_{sc}

Short-circuit current, including that not inhibited by Bt, was promptly and reversibly inhibited by oxygen lack, as shown in Fig. 3. When oxygen was replaced by nitrogen in the absence of Bt, the short-circuit current dropped to near zero, and was restored to the level to be expected (after a normal rate of decline) when oxygen was restored. Subsequently, full Bt inhibition was induced by 1 IU/ml Bt on the lumen side. Replacement of oxygen by nitrogen then caused a reversible inhibition of the remainder of the short-circuit current, during which the I_{sc} transiently reached a negative value, suggesting a transient net flux of positive ions from lumen-side to blood-side, before complete cessation of active ion transport.

Effects of Bt on potassium influx, $J_{K(BL)}$

As shown in Fig. 4, addition of Bt on the lumen side to a concentration of 2 IU/ml, which strongly inhibited the I_{sc}, had no effect on the unidirectional flux of potassium from blood-side to lumen-side.

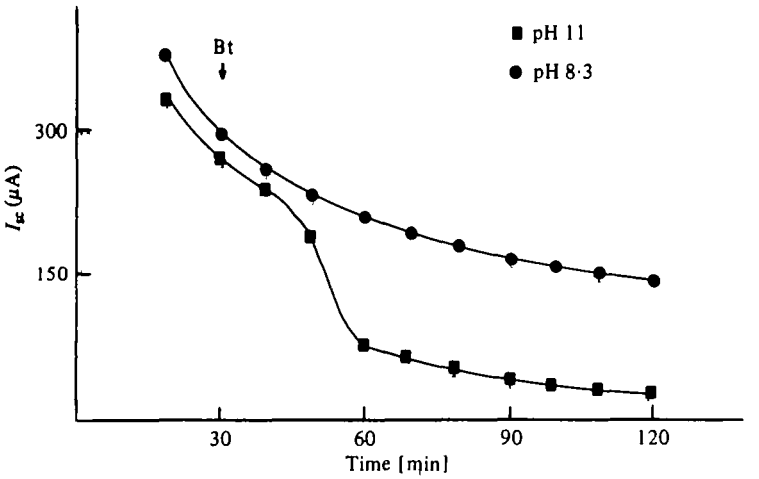

Fig. 1. Effect of solution pH on inhibitor activity of Bt. Bt (2 IU/ml) was without effect on a midgut which was bathed on both sides by pH 8·3 solution (●). However, within 40 min after addition the Bt had caused a 70% inhibition of the I_{sc} of a midgut whose lumen-side was bathed by pH 11 solution (■). During the second hour of the experiment no additional effects of Bt were observed. I_{sc} was recorded at 10 min intervals. Lines were fitted through the points by inspection.

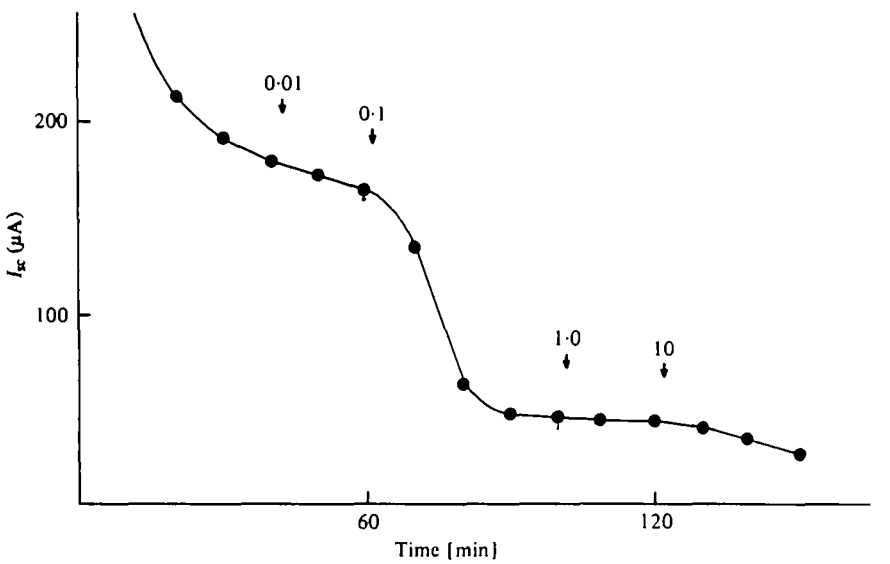

Fig. 2. Effect of concentration of pre-activated Bt on I_{sc}. 0·01 IU/ml pre-activated Bt (see text) had no effect on I_{sc}, however, 0·1 IU/ml caused a 67% decrease. Increasing the Bt concentration on the lumen-side to 1 and 10 IU/ml led to no further inhibition. I_{sc} was recorded at 5 min intervals. The line was fitted through the points by inspection.

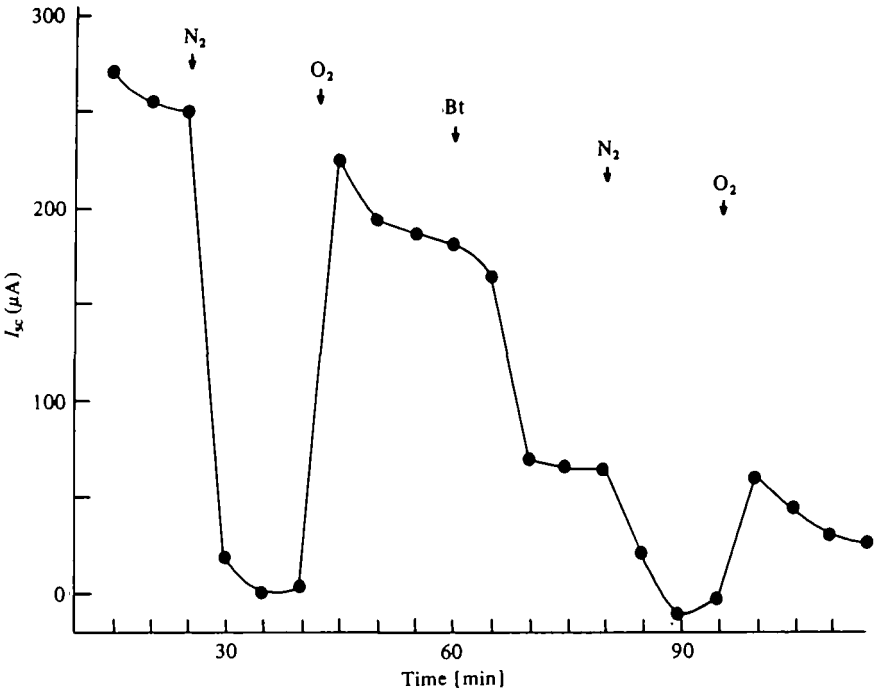

Fig. 3. Effects of anoxia on I_{sc}. Replacement of nitrogen for oxygen caused a reversible inhibition of the I_{sc} both before and after inhibition by 1 IU/ml Bt. Note the transient 'overshoot' of the I_{sc} when oxygen was restored and the transient negative values of I_{sc} during anoxia after Bt inhibition. I_{sc} was recorded at 5 min intervals. The line was fitted by inspection.

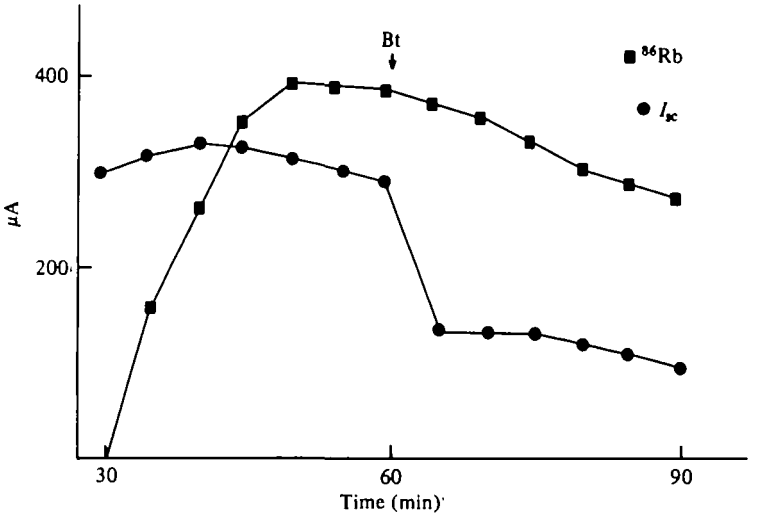

Fig. 4. Effect of Bt on potassium influx. After achievement of the tracer steady state (Wood & Harvey, 1975), 2 IU/ml Bt caused inhibition of I_{sc} (●) but had no effect on the unidirectional flux of potassium (■) from blood-side to lumen-side. Measurements were made at 5 min intervals. Lines fitted by inspection.

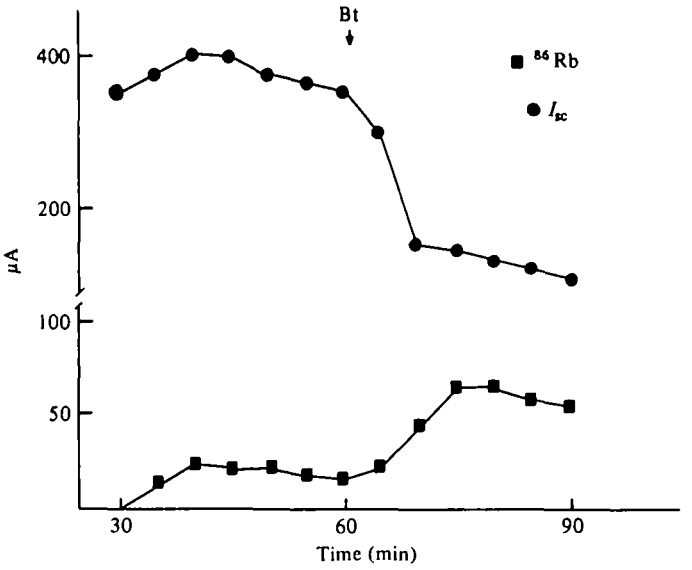

Fig. 5. Effect of Bt on potassium efflux. After achievement of the tracer steady state (Wood & Harvey, 1975), 2 IU/ml Bt on the lumen-side caused inhibition of the I_{sc} (●) and increased the unidirectional flux of potassium (■) from lumen-side to blood-side. Measurements were made at 5 min intervals and lines were fitted by inspection.

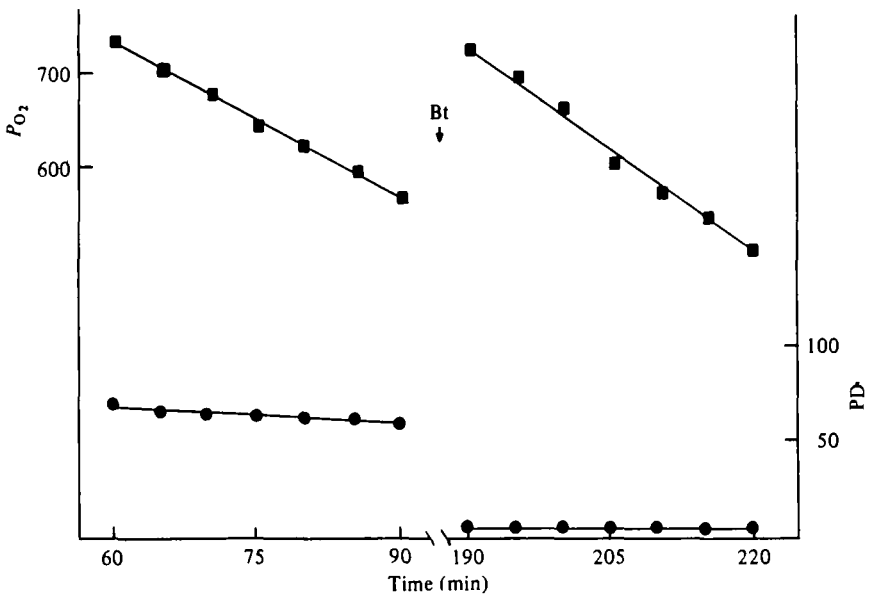

Fig. 6. Effects of Bt on oxygen uptake. After allowing 1 h for the potential difference (PD) to stabilize and the bathing solutions to become completely saturated with oxygen, the blood-side was sealed and the oxygen pressure (■), in mmHg, as well as the PD (●), in mV, was recorded at 5 min intervals for 30 min. During this period the P_{O_2} decreased at a rate of 5·33 mmHg per minute and the PD decayed at a rate of 0·2 mV/min. At 90 min the bathing solution was again saturated with oxygen and at 141 min Bt was added to the lumen-side of the chamber to give a concentration of about 2 IU/ml. PD then decreased and was allowed to stabilize at a new inhibited value before the blood-side of the chamber was again sealed at 190 min. During the period from 190 to 220 min the oxygen pressure decreased at a rate of 7·00 mmHg per minute while the PD decayed from 5·5 to 5·0 mV. Thus the rate of oxygen uptake by the isolated midgut increased by 31% in the presence of Bt. The lines were fitted by linear regression.

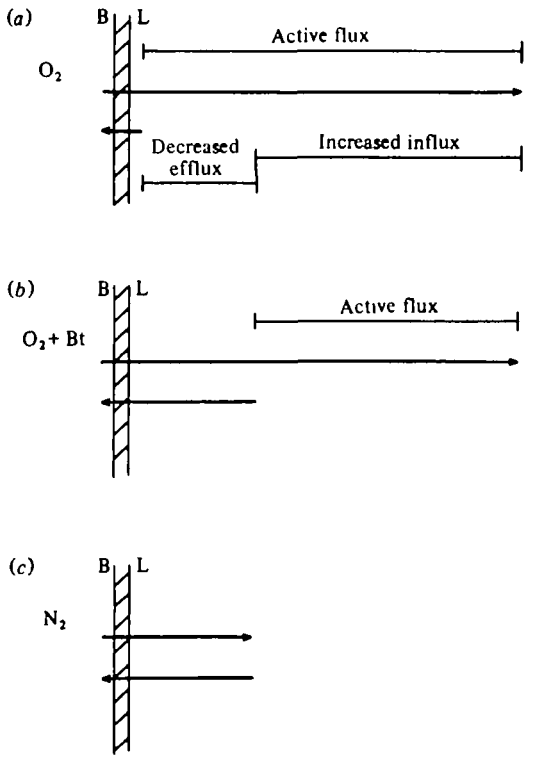

Fig. 7. Effect of Bt on the potassium transport mechanism. (*a*) In the presence of oxygen potassium influx from blood (B) to lumen (L) is increased and potassium efflux from L to B is decreased, compared to passive movements (*c*), resulting in a large net 'active' flux. (*b*) Bt, in the presence of oxygen, abolishes the decrease in the efflux with a corresponding decrease in the net flux. (*c*) In nitrogen, in the absence of active transport, fluxes from B to L and L to B are equal. According to this hypothesis the action of Bt is to disrupt a coupling of the metabolism to potassium efflux while sparing that to the influx.

Effects of Bt on potassium efflux, $J_{K(LB)}$

The potassium flux from lumen-side to blood-side in the presence of 2 IU/ml of Bt on the lumen-side was 2·9 times (S.E. 0·7; $n = 3$) greater than that in the absence of Bt. This stimulation of the efflux was large enough to account for 29% (S.E. 5; $n = 3$) of the decrease in I_{sc}. The stimulation followed a time-course similar to that of the inhibition of the short-circuit current (Fig. 5).

Effects of Bt on electrical resistance, R_m

In 26 experiments the mean resistance measured in the absence of Bt was $230 \pm 190\ \Omega\ cm^2$; in the presence of Bt it was $135 \pm 116\ \Omega\ cm^2$. The ratio, R_m in Bt/R_m, between values taken in pairs was $0{\cdot}54 \pm 0{\cdot}23$.

Effects of Bt on oxygen uptake

Despite the technical limitations (Materials and Methods) the data in Fig. 6 strongly suggest a stimulation of oxygen uptake by Bt, from a value of 5·33 to 7·00 mm-

Hg/min; an increase of 31%. Normally the rate of oxygen uptake, like the rate of potassium transport, decreases with time. Moreover Mandel, Riddle & Storey (1979*b*) have found that the concentration of ADP is high in midgut tissue and normally drives oxygen uptake at nearly maximal values. Both of these observations tend to increase the significance of the present result.

DISCUSSION

Activation of Bt at high pH

The effects of *Bacillus thuringiensis* spores and endotoxin treated at high pH upon the isolated midgut of *Manduca sexta* are in accord with the observations of Faust *et al.* (1974*a*) that the non-toxic crystalline protein (MW 230000 daltons; Holmes & Monro, 1965) is activated by enzymatic and alkaline hydrolysis in the insect gut with the production of toxic peptides (MW 1000–30000 daltons). The inhibitory concentration amounts to ca 10^{-10} M assuming that there is one active site per protein molecule. The effects produced by the toxin upon the midgut indicate that a specific component of active potassium transport is inhibited. After treatment with the toxin the isolated midgut still has some potassium transport function, since Bt has no effect on 40% of the short-circuit current (Figs. 1, 2) and no effect on the potassium influx (Fig. 4). This partial inhibition might have resulted from incomplete digestion of Bt *in vitro* but this explanation seems unlikely since increasing Bt concentrations by 100-fold did not increase inhibition (Fig. 2). A more likely explanation is that the toxin inhibits only one of two potassium transport sites.

Mechanism of action of Bt

The observation that Bt has no effect, either stimulatory or inhibitory, on potassium influx toward the lumen (Fig. 4) allows us to rule out all explanations which require a *decrease* in the influx (e.g. inhibition of active influx toward the lumen), and all of those which require an *increase* in the influx (e.g. increased exchange diffusion, increased facilitated diffusion via a potassium ionophore, and a general permeability increase). Although the resistance decrease in Bt suggests a general permeability increase, it is equally well explained by a specific increase in potassium efflux (Fig. 5). A further argument against a general permeability increase, and also an argument against an effect on active ion transport in general, is the failure of the toxin to abolish the negative current associated with calcium and magnesium active transport toward the blood-side of the midgut (e.g. Fig. 3; Griego *et al.* 1979).

Equally unlikely are all explanations which require an unequal flux of potassium from the tissue to the blood-side and lumen-side bathing solutions. Assuming a cellular K^+ concentration of 100 mM (Zerahn, 1978), an extracellular K^+ concentration of 32 mM (Materials and Methods), an extracellular space of 49% (Abramcheck, Blankemeyer & Harvey, 1979), and midgut weight of 22 mg (Materials and Methods), the total tissue potassium amounts to less than 2 μequiv. This is sufficient to account for the total unidirectional fluxes, which amount to 15 μequiv/h (Results), for no more than 10 min, whereas these fluxes persist for hours. On these grounds we can eliminate explanations of Bt action such as a passive short-circuit current (Rehm,

1968) carried by potassium from cells to blood-side or dissipation of gradients between cell interior and blood-side by exchange or facilitated diffusion.

Bt allows the experimental separation of the short-circuit current and its equivalent, the net potassium flux toward the lumen (the active potassium transport) into two components, both of which are inhibited by oxygen lack but only one of which is inhibited by Bt (Fig. 3). Bt stimulates the efflux (Fig. 5) but has no effect, either stimulatory or inhibitory, on the influx (Fig. 4). Therefore Bt must act on an active (oxygen dependent) component of the potassium efflux.

An hypothesis which accounts for all the experimental observations is diagrammed in Fig. 7. In oxygen the net flux has two active components, an active increase in the influx and an active depression of the efflux (Fig. 7*a*). Bt inhibits the active depression of the efflux thereby decreasing the net flux (Fig. 7*b*). Similarly, oxygen-lack inhibits the active depression of the efflux. Thus Bt and nitrogen both undo the effects of oxygen on the efflux. But nitrogen also inhibits the active increase in the influx. The net result is that influx and efflux are equal in nitrogen and there is no net flux (Fig. 7*c*). This hypothesis is supported by the experimental observations (1) that the ratio of efflux in oxygen to that in nitrogen is 0·3 (Blankemeyer, 1978; confirmed in unpublished work by M. Cioffi in this laboratory) and (2) that the ratio of efflux in oxygen to that in O_2 + Bt is 0·3 (Results). Therefore, the ratio of efflux in oxygen plus Bt to that in nitrogen is 1. Moreover, Blankemeyer (1978) showed that under conditions where potassium, but not caesium, is actively transported, only the efflux of potassium, and not of caesium, is depressed by oxygen and concluded that the enhanced potassium efflux in nitrogen is through the potassium pump.

The evidence that both oxygen lack and Bt inhibit an active depression of the efflux is convincing qualitatively. However, neither the increased efflux in nitrogen nor that in Bt account for more than 30% of the corresponding inhibition of the short-circuit current. Further detailed study of the effects of oxygen lack and Bt on the potassium influx and their effects on active calcium transport (Wood & Harvey, 1976) and active magnesium transport (Wood, Jungreis & Harvey, 1975) toward the blood-side may prove rewarding.

Primary action of Bt

The stimulation of oxygen uptake in the isolated midgut by Bt (Fig. 6) is reminiscent of the stimulation of mitochondrial oxygen uptake by Bt (Travers *et al.* 1976) which led us to consider initially that the effects of Bt on potassium transport might be secondary to its uncoupling of oxidative metabolism as argued by Faust and coworkers (review by Faust & Travers, 1979). Their arguments rest heavily on the finding by Fast & Donaghue (1971) that Bt stimulates glucose uptake within 1 min after administration *per os* to the living insect. The effects of Bt on the short-circuit current are sometimes observed in less than 5 min (e.g. Figs. 2, 4, 5) but these experiments were not designed to measure this lag time, which is variable in our data. The lag time for effects of Bt on K efflux is hard to define with present sampling procedures. Therefore one cannot make a conclusive choice as to whether the oxidative uncoupling action or the action on potassium transport is the primary one on the basis of present kinetic evidence.

However, several objections can be raised to the oxidative uncoupling hypothesis. A general uncoupler of oxidative metabolism could not leave 40% of the short-circuit current and the entire active potassium influx (Fig. 4) unaffected but still reversibly inhibitible by oxygen lack (Fig. 3). Likewise, it could not spare general body movements and heartbeat, yet these are observed for many minutes after Bt has strongly inhibited glucose uptake and potassium efflux. Equally unexplained is the observation that only the 30000-dalton peptide uncouples oxidative phosphorylation (Travers *et al.* 1976) whereas the smaller components, such as the 5000-dalton fraction, are toxic and stimulate oxygen uptake (Faust, Travers & Hallam, 1974*b*) but do not uncouple oxidative phosphorylation (Travers *et al.* 1976).

Bt as K pump inhibitor

The analysis of the midgut K pump has been hampered from the start by the lack of a specific inhibitor. Carbonic anhydrase inhibitors and agents which act on oxidative metabolism and glycolysis do inhibit the short-circuit current (Haskell, Clemens & Harvey, 1965; Mandel *et al.* 1979*a*) but have found limited usefulness in transport studies. Recently, Zerahn & Koefoed (1979) have described what may be a facilitated diffusion system for thallium ions in the Cecropia midgut and have reported that thallium appears to be a specific inhibitor of the potassium pump. The high potency of Bt (10^{-10} M for full 60% inhibition) and the irreversible nature of its inhibition suggest that Bt may be a useful tool for further studies of active potassium transport both in the midgut and in other insect systems such as Malpighian tubules.

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