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# CHANGES IN MIDGUT ACTIVE ION TRANSPORT AND METABOLISM DURING LARVAL–LARVAL MOLTING IN THE TOBACCO HORNWORM (*MANDUCA SEXTA*)

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## Summary

**Ion transport and metabolism in the posterior midgut before, during and after the molt to the fifth instar of the tobacco hornworm *Manduca sexta* were investigated. *In situ* measurements reveal that the transepithelial potential difference of the posterior midgut falls during the molting process. This finding was confirmed by *in vitro* experiments in which it was demonstrated that both the transepithelial potential and the short-circuit current are lower in molting fourth instars compared with feeding fourth instars. The short-circuit current increases after ecdysis, with a maximal rate being achieved approximately 4 h after the molt. Resumption of feeding after the molt is not necessary to initiate this increase in active ion transport. The metabolic organization of the tissue also changes during the molting process. The maximal activities of glycolytic enzymes and 3-hydroxyacyl-CoA dehydrogenase, an enzyme of lipid β-oxidation, decrease during the molting process and increase after ecdysis. Although citrate synthase activity, an index of maximal aerobic capacity, decreases during the molt and increases again after ecdysis, tissue respiration is the same in feeding fourth instars and molting larvae. This result indicates that a greater percentage of maximal aerobic capacity is used during molting and that energy may be diverted to cell proliferation and differentiation and away from the support of active ion transport at this time.**

Key words: *Manduca sexta*, tobacco hornworm, ecdysis, ion transport.

## Introduction

Like all caterpillars, the tobacco hornworm (*Manduca sexta*) grows rapidly, increasing in mass 10 000-fold in 16 days (Goodman *et al.* 1985). The midgut, which transports $K^+$ (Cioffi and Harvey, 1981; Wolfersberger and Giangiacomo, 1983; Moffett and Koch, 1985; Chamberlin, 1990*a*), produces an alkaline secretion (Chamberlin, 1990*b*) and digests and absorbs nutrients (reviewed by Dow, 1986), must also increase in size as the larva grows. The midgut goblet cells create the large $K^+$ and pH gradients across the midgut, while the columnar cells are thought to absorb nutrients (reviewed by Dow, 1986). Baldwin and Hakim (1991) have described how new cells are added to the midgut during the molt, a process which occurs over a 24 h period between apolysis (separation of the new and old cuticle) and ecdysis (shedding of the exoskeleton; Goodman *et al.* 1985). After apolysis, but before ecdysis, stem cells differentiate into goblet and columnar cells in such a way that new cells interdigitate between existing cells so that the ratio of five columnar cells to one goblet cell is maintained.

Because the number of midgut cells increases fourfold during each larval molt (Baldwin and Hakim, 1991), 75 % of the cells in any one instar are those produced during the preceding molt. Therefore, epithelial function will be greatly influenced by the properties of these newly formed cells. There have been only a few studies, however, that have addressed changes in midgut function during molting. In a study by Sumner *et al.* (1995) and a preliminary report by Gibellato and Chamberlin (1994*a*), it was found that the transepithelial potential difference (PD) decreases during the molt from the fourth to the fifth instar. This decline in PD is paralleled by a decrease in $H^+$-ATPase activity (Sumner *et al.* 1995) as well as citrate synthase (CS) activity, an indicator of aerobic capacity (Gibellato and Chamberlin, 1994*a*). These results indicate that both the proton pump, which is responsible for establishing a large membrane potential across the apical membrane of the goblet cells (reviewed by Klein, 1992), and aerobic metabolism, which is necessary for supplying ATP for the proton pump, are depressed during the molt. No study, however, has demonstrated whether there are changes in the rate of epithelial active ion transport during and after ecdysis and whether these changes are accompanied by a metabolic reorganization of the tissue. In the present study, changes in posterior midgut ion transport and metabolism were studied before, during and after the molt from the fourth to the fifth instar in tobacco hornworms. Changes in midgut PD were measured *in situ* in feeding fourth instars, pharate fifth instars

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and feeding fifth instars. In addition, the short-circuit current ($I_{sc}$) and PD were measured in midguts dissected from larvae before, during and at several times after ecdysis. Metabolic changes were assessed by measuring midgut oxygen consumption and by monitoring maximal enzyme activities. Hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK) activities were used as indices of glycolytic activity. Lactate dehydrogenase (LDH) activity was measured to assess changes in anaerobic metabolism, whereas CS activity was used as an index of aerobic metabolism. Glutamate dehydrogenase (GDH) is a key enzyme in amino acid metabolism, and lipid oxidation was assessed by measuring the activity of 3-hydroxyacyl-CoA dehydrogenase (HOAD). Arginine kinase (AK) was measured since it may be involved in regulating midgut mitochondrial metabolism (Chamberlin *et al.* 1994).

## Materials and methods

### *Rearing and staging of insects*

*Manduca sexta* (Johansson) larvae were raised from eggs provided by the United States Department of Agriculture (USDA) or a from colony derived from the USDA stock and maintained in the Biological Sciences Department at Ohio University, USA. Larvae were fed a commercial diet (no. 9783, BioServ, Frenchtown, NJ, USA) and maintained at 25 °C on a 16 h:8 h L:D cycle.

Fourth- and fifth-instar larvae were used in this study. Feeding fourth instars weighed between 0.9 and 1.4 g and showed no signs of preparation for molting (e.g. gut purge, head capsule slippage). In order to facilitate comparisons between the findings of the present study and the morphology of the midgut, pharate fifth instars at three stages described by Baldwin and Hakim (1991) were used: the head capsule was slipped, green, but clear at its base (stage D); the head capsule was opaque with the white mandibles of the fifth instar visible (stage E); black fifth-instar mandibles were visible through a clear head capsule (stage F). For studies using fifth instars, molting larvae (stage F) were placed in the compartments of an ice-cube tray with or without food present. Ecdysis was recorded using a Hitachi camcorder (model VM-1700A) suspended in the biological incubator. The precise time of ecdysis was noted by reviewing the video tape on a Mitsubishi video cassette recorder (model HS-U28) using a Craig television monitor (model PC 1343). Shedding of the exoskeleton takes several minutes and, therefore, ecdysis was defined as the time when the exuvium was sloughed over the thorax.

### *Electrophysiological measurements*

*In situ* midgut PD was monitored by employing a slight modification of the method described by Moffett and Cummings (1994). Feeding fourth instars, pharate fifth instars (stage F) or fifth instars (24 h after ecdysis) were chilled on ice for 10–20 min. The anterior end was then ligated with a thread just tightly enough to prevent loss of gut material when the animal was subsequently decapitated. Although the anterior end was ligated, an agar bridge (PE20 tubing filled with 3 mol l$^{-1}$ KCl and 4 % agar) could still be inserted into the gut through the anterior opening and advanced to the level of the posterior midgut. A second, and tighter, ligature was then tied around the anterior end of the animal to ensure that the agar bridge would remain in position. A second agar bridge was inserted into the hemocoel near the horn at the posterior end of the animal, and the exposed portion of the tubing was tied to the horn with a thread. The PD was measured using calomel electrodes connected to the free ends of the agar bridges by a KCl solution. PD was monitored using an epithelial voltage-clamp (World Precision Instruments, DVC 1000) and recorded using a computer data acquisition system (AcqKnowledge, version 3.1). After recording the PD, the animal was dissected and the location of the agar bridges was confirmed.

$I_{sc}$ and PD were measured *in vitro* using a modified version of the method described by Chamberlin (1994). Posterior midguts were dissected from larvae and mounted in modified Ussing chambers (Hanrahan *et al.* 1984) with a 0.0636 cm$^2$ opening. The tissue was bathed bilaterally with saline (Chamberlin, 1994) and bubbled with 100 % $O_2$. The PD and $I_{sc}$ across the posterior midgut were measured as described by Chamberlin (1994) except that data were recorded with a computer data acquisition program, Datacan V (Sable Systems, Inc., version 1.0). Every 15 min, the voltage-clamp was turned off and the PD, which took 3 min to stabilize, was allowed to develop. Resistance ($\Omega$ cm$^2$) was calculated from this PD and the $I_{sc}$ measured just before turning off the voltage-clamp. The $I_{sc}$ was measured for a total of 1 h. To check that there were no leaks in the epithelium, amaranth dye was added to one side of the Ussing chamber after the measurement period. If dye appeared on the opposite side of the chamber within 5 min, the data were discarded.

### *Tissue respiration*

Small portions (less than 6 mg) of posterior midgut were dissected from the larvae, rinsed in saline, blotted dry and weighed. The tissue was then placed in 5 ml of saline, which was continuously oxygenated and incubated for 30–40 min at 25 °C. After this preincubation period, the tissue was transferred to a 0.9 ml temperature-controlled (25 °C) chamber containing fresh oxygenated saline, and the rate of oxygen consumption was measured using a Clark-type electrode. The tissue was gently stirred during the respiration measurements. Potassium cyanide (final concentration, 1 mmol l$^{-1}$) was added to the solution bathing the tissues to confirm that oxygen consumption was due to mitochondrial respiration. Possible damage to the tissue during the respiratory measurements was assessed by monitoring enzyme leakage from the tissue. The activity of phosphoglucoisomerase (PGI), a cytosolic enzyme, was measured in both the tissue and saline in the respiration chamber. The activity of PGI was measured according to the method described by Chamberlin and Phillips (1983) except that 50 mmol l$^{-1}$ imidazole (pH 7.2) was used.

### *Enzyme assays*

The tissue was prepared for enzymatic analysis using a method similar to that described by Chamberlin (1994). The midgut was dissected from the larva, rinsed in 32K saline (Cioffi and Harvey, 1981), blotted dry and weighed. The tissue was then homogenized in 50 mmol l$^{-1}$ imidazole buffer, pH 7.2. Homogenates used in the measurement the activities of HK, PFK, PK, LDH, GDH, HOAD and AK were centrifuged at 20 000 ***g*** for 20 min and the supernatants were reserved for analysis. Triton X-100 (final concentration 0.1 %) was added to homogenates that would be used for the measurement of CS activity, the homogenate was sonicated, centrifuged at 20 000 ***g*** for 20 min, and the supernatant analyzed for enzyme activity. The activities of PK, LDH, GDH, HOAD, AK and CS were measured according to methods described previously (Chamberlin, 1994; Gibellato and Chamberlin, 1994*b*). HK activity was measured using a method modified from that of Chamberlin and Phillips (1983) in which 50 mmol l$^{-1}$ imidazole buffer (pH 7.2) replaced the Tris buffer. The assay conditions for PFK were as follows: 50 mmol l$^{-1}$ imidazole (pH 7.2), 10 mmol l$^{-1}$ $MgCl_2$, 50 mmol l$^{-1}$ KCl, 0.15 mmol l$^{-1}$ NADH, 2 mmol l$^{-1}$ ATP, 0.025 mmol l$^{-1}$ fructose 2,6-bisphosphate, excess triosephosphate isomerase, excess aldolase and 5 mmol l$^{-1}$ fructose 6-phosphate (omitted in control). Activities were measured, in duplicate, on a Gilford Response spectrophotometer at 25 °C.

### *Statistics*

Values are expressed as means ± standard errors (S.E.M.) with *N* indicating the number of midguts measured. Statistical analyses were conducted using an analysis of variance (ANOVA) and, when appropriate, a *post-hoc* test (least significant difference). $P\leqslant 0.05$ was considered to represent a significant difference.

## Results

The midgut PD, measured *in situ*, is stable for at least 30 min (Fig. 1). Injection of 10 µl of 100 mmol l$^{-1}$ KCN into the hemocoel abolished the PD (data not shown). The midgut PD of pharate fifth instars was significantly lower than that of feeding fourth instars (Fig. 1). Similarly, the midgut PD, measured *in vitro*, was lower in larvae at various stages of molting than in feeding fourth instars (Table 1). The midguts of feeding fourth-instar larvae also had a higher $I_{sc}$ than those of all pharate fifth instars (Fig. 2). The differences in $I_{sc}$ shown in Fig. 2 were not due to differences in tissue fragility since the $I_{sc}$ was maintained at a steady rate and the resistances did not differ significantly between stages (Table 1). It should be noted that in this study $I_{sc}$ and PD measurements were obtained using an Ussing chamber with an orifice that was smaller than that used previously (Chamberlin, 1994). It appears, however,

Table 1. In vitro *midgut transepithelial potentials and resistances*

| | Feeding fourth instars | Pharate fifth instars | | |
|---|---|---|---|---|
| | | Stage D | Stage E | Stage F |
| Potential difference (mV) | 75.5±2.8 | 53.0±3.8* | 42.2±3.4* | 42.6±2.5* |
| Resistance (Ω cm$^2$) | 41.9±2.5 | 54.1±7.5 | 51.9±7.1 | 68.5±11.5 |

Values are means ± S.E.M., *N*=8–16.

* denotes a significant difference from the values obtained for feeding fourth instars.

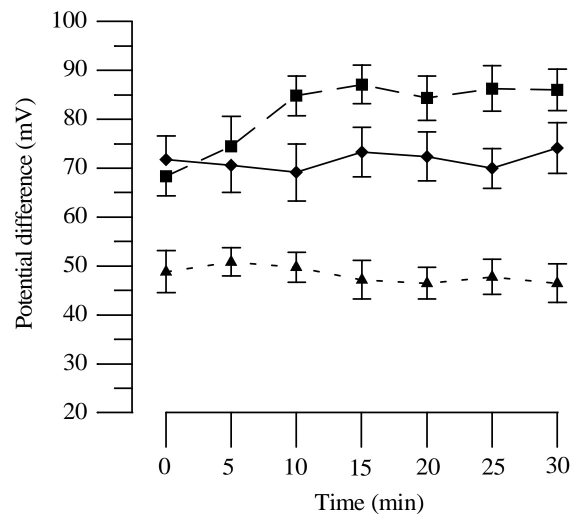


Fig. 1. Changes in *in situ* midgut potential difference over time after cannulation of the hemocoel (see Materials and methods): (◆) feeding fourth instars, *N*=8; (▲) pharate fifth instars, stage F, *N*=8; (■) fifth instars, 24 h after ecdysis, *N*=8. Values are means ± S.E.M.

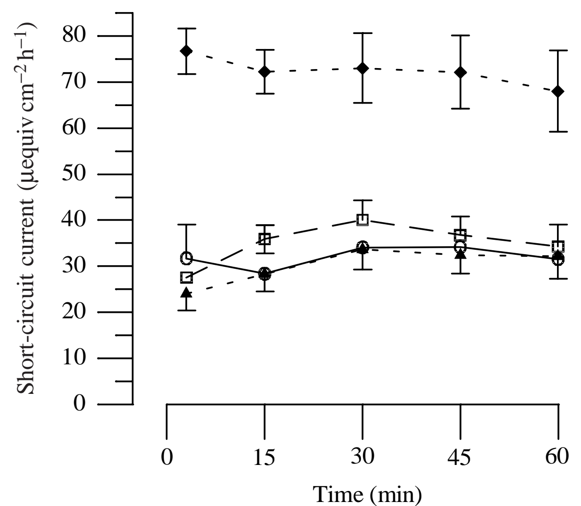


Fig. 2. Changes in fourth-instar short-circuit current over time after mounting the midgut tissues in the Ussing chambers: (◆) feeding fourth instars, *N*=16; (□) pharate fifth instars, stage D, *N*=8; (○) pharate fifth instars, stage E, *N*=9; (▲) pharate fifth instars, stage F, *N*=14. Values are means ± S.E.M.

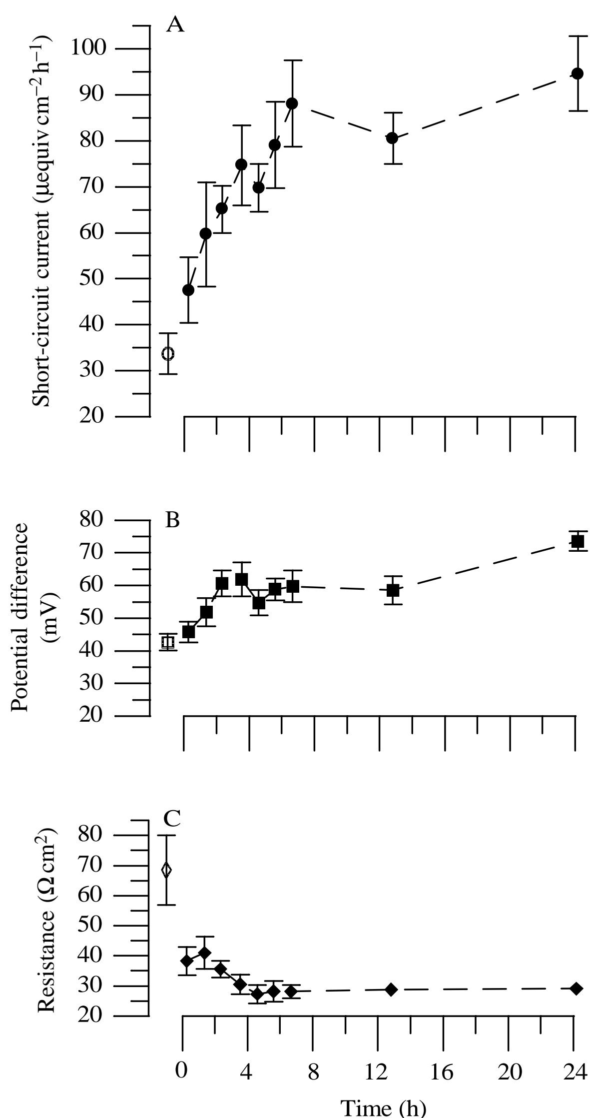


Fig. 3. Changes in midgut short-circuit current (A), transepithelial potential difference (B) and resistance (C) after ecdysis. Short-circuit current and transepithelial potential values are those taken 30 min after mounting the tissues in the Ussing chambers. Data for pharate fifth instars (stage F) are shown by open symbols. Values are means ± S.E.M., $N$=7–9.

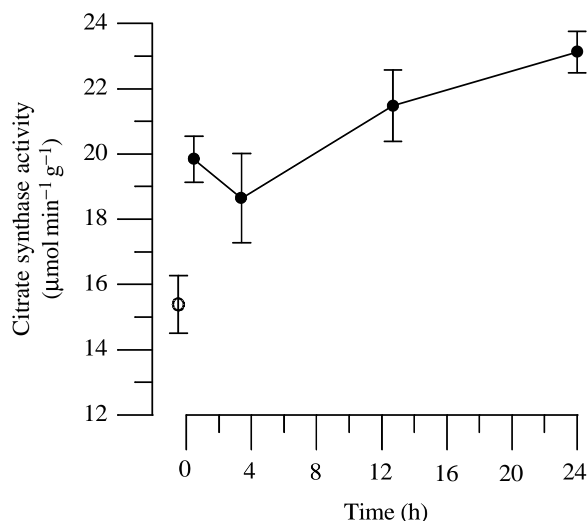


Fig. 4. Changes in citrate synthase activity after ecdysis. The value for pharate fifth instars (stage F) is shown by the open symbol. Values are means ± S.E.M., $N$=6–7.

that the smaller opening did not lead to increased edge damage since the values for $I_{sc}$ and PD of fifth-instar larvae, 24 h after ecdysis (Fig. 3), were similar to those reported in the study by Chamberlin (1994). Furthermore, the PDs measured *in situ* (Fig. 1) were similar to those measured *in vitro* (Table 1; Fig. 3), indicating that the reported *in vitro* measurements reflect the conditions within the animal.

After ecdysis, there was a rise in $I_{sc}$ and PD and a decline in resistance, with the maximal $I_{sc}$ being achieved approximately 4 h after ecdysis (Fig. 3). Feeding was not needed to stimulate active ion transport since the $I_{sc}$ was identical in midguts taken from fed (74.6±8.7 μequiv cm$^{-2}$ h$^{-1}$, $N$=7) and unfed (79.1±16.6 μequiv cm$^{-2}$ h$^{-1}$, $N$=7) larvae 3.5 h after ecdysis.

The respiration rates of the midguts of feeding fourth instars (1.78±0.08 nmol $O_2$ min$^{-1}$ mg$^{-1}$, $N$=19) and pharate fifth instars (stage F; 1.93±0.08 nmol $O_2$ min$^{-1}$ mg$^{-1}$, $N$=21) were not significantly different. The midguts of fifth-instar larvae, 24 h post-ecdysis, have a respiration rate (2.20±0.08 nmol $O_2$ min$^{-1}$ mg$^{-1}$, $N$=20) significantly greater than those of feeding fourth instars and pharate fifth-instar stage F larvae. Differential tissue damage incurred during the measurement period cannot account for differences in respiration since the loss of PGI activity was the same for midguts from all three stages of development (feeding fourth instars, 3.5±0.7 %, $N$=6; pharate fifth-instar stage F larvae, 4.2±0.6 %, $N$=5; fifth instars, 5.4±0.6 %, $N$=6). Furthermore, the differences in respiration reflect differences in mitochondrial respiration since the percentage inhibition of respiration by 1 mmol l$^{-1}$ KCN was the same in all of these preparations (feeding fourth instars, 78.7±1.9 %, $N$=7; pharate fifth-instar stage F larvae, 77.0±2.2 %, $N$=9; fifth instars, 76.6±2.4 %, $N$=9).

During the process of molting, LDH and GDH activities did not change, but there was a significant decline in the activities of HK, PFK, PK, HOAD and CS (Table 2). CS activity increased rapidly after ecdysis (Fig. 4; Table 2). 24 h after ecdysis, the activities of HK, PK, CS and HOAD were the same as those in feeding fourth instars (Table 2). Only GDH and AK activities were lower in fifth instars compared with activities in feeding fourth instars.

## Discussion

During larval–larval molting, new cells are added to the midgut of *M. sexta* (Baldwin and Hakim, 1991), and the results of the present study indicate that these morphological changes are paralleled by changes in epithelial ion transport and metabolism. It appears that active ion transport is maintained

Table 2. *Midgut enzyme activities in fourth- and fifth-instar tobacco hornworms* Manduca sexta

| Enzyme | Activity (μmol min$^{-1}$ g$^{-1}$) | | |
|---|---|---|---|
| | Fourth instars | Pharate fifth instars | Fifth instars |
| Hexokinase (*N*=6–8) | 6.1±0.3 | 4.9±0.3* | 6.2±0.4† |
| Phosphofructokinase (*N*=8) | 1.1±0.1 | 0.4±0.1* | 3.2±0.1*,† |
| Pyruvate kinase (*N*=14) | 23.5±0.9 | 14.5±0.6* | 23.6±1.4† |
| Lactate dehydrogenase (*N*=14) | 7.0±0.4 | 7.5±0.3 | 8.3±1.3 |
| Arginine kinase (*N*=14) | 65.4±3.7 | 53.1±7.9 | 41.5±3.0* |
| Glutamate dehydrogenase (*N*=8) | 7.9±0.7 | 9.6±0.8 | 4.6±0.3*,† |
| 3-Hydroxyacyl-CoA dehydrogenase (*N*=8) | 7.0±0.6 | 4.9±0.5* | 5.5±0.7 |
| Citrate synthase (*N*=14) | 24.8±0.6 | 17.7±0.9* | 24.4±0.7† |

The data for the pharate fifth instars are from stage F larvae. The data for the fifth instars were obtained from larvae 24 h after ecdysis.

* denotes a significant difference from the value for the fourth instar; † indicates a significant difference from the value for the pharate fifth instar.

Values are means ± S.E.M.

during molting, albeit at a lower rate than in feeding larvae. This finding is in contrast to the study of Sumner *et al.* (1995), in which it was reported that the *in vitro* PD of the middle region of the midgut fell to zero, and even to negative values, during molting. The disparity between the findings in the two studies may reflect regional differences in midgut transport during molting. Alternatively, the difference between the PD measured in the study by Sumner *et al.* (1995) and that measured in the present study may reflect the different salines used in the two studies. Sumner *et al.* (1995) employed a KCl-based saline which contained a few metabolic substrates, whereas the present study employed a more complex saline containing hemolymph levels of ions, including $Na^+$, and a wide variety of organic solutes. This more complex saline may have maintained some *in vitro* transport processes more effectively in the midguts of molting larvae.

Changes in epithelial ion transport during molting may be due to changes in vacuolar-type ATPase (V-ATPase) activity. Sumner *et al.* (1995) showed that goblet cell membranes isolated from the posterior midguts of molting larvae (stage E) had a V-ATPase activity that was only 15 % of that of membranes isolated from feeding fifth instars, and that this reduction in activity was correlated with the absence of subunit B in mature and newly formed goblet cells (Sumner *et al.* 1995). Immunoreactivity to subunit A, however, is detectable in both old and new goblet cell apical membranes in stage F larvae (Jäger *et al.* 1996). Since both stage E and stage F larvae have identical low values of $I_{sc}$, it is difficult to correlate changes in ion transport with the presence or absence of specific V-ATPase subunits.

Pharate fifth instars at stage F have a reduced CS activity, indicating a reduction in aerobic capacity. This reduction in CS activity corresponds with the observation that newly formed midgut cells in stage F larvae have fewer, smaller and paler mitochondria than the older cells (Baldwin and Hakim, 1991). Since 75 % of the epithelium is composed of these newly differentiated cells, the lower mitochondrial content may account for the lower CS activity measured in the entire epithelium. Despite a diminished maximal aerobic capacity, there was no difference in the rate of oxygen consumption of the intact tissues dissected from larvae before and during the molting process. Although there is always the possibility that *in vitro* measurements of tissue oxygen consumption do not completely reflect the situation *in vivo*, these data seem to indicate that, during molting, the tissue is using a greater percentage of its aerobic capacity. Furthermore, during this period of development, the mitochondrial ATP production may be diverted from supporting active ion transport to supporting the production and differentiation of new cells, leading to a diminished rate of active ion transport.

Active ion transport in the tobacco hornworm midgut is supported by the oxidation of carbohydrates and lipids (Chamberlin, 1987, 1989). During molting, when active ion transport is depressed, there is a concomitant decrease in the activities of glycolytic enzymes and in the activity of an enzyme of fatty acid oxidation, HOAD. The fall in HOAD activity during molting also correlates with the observed accumulation of lipid droplets in the mature columnar cells (Baldwin and Hakim, 1991).

Within 1 h after ecdysis, there is a substantial increase in CS activity, but the activity continues to rise over the subsequent 23 h. In addition, in the present study, tissue respiration was greatest 24 h after ecdysis. These results indicate that adjustments to aerobic metabolism, perhaps due to processes such as mitochondrial replication, mitochondrial protein synthesis or protein importation, continue to take place after cellular differentiation has been completed. It is also important to note that maximal aerobic capacity was reached several hours after the maximal $I_{sc}$ was attained. This comparison demonstrates that active ion transport is not limited by the aerobic capacity of the tissue. Instead, it must be regulated by other processes, such as the partitioning of ATP among various energy-using processes or the synthesis and assembly of transport proteins (Sumner *et al.* 1995; Jäger *et al.* 1996).

The activities of arginine kinase and glutamate dehydrogenase in fifth-instar larvae reported in the present study are approximately 50 % lower than those reported for midguts of fifth instars 60 h post-ecdysis (Chamberlin, 1994; Gibellato and Chamberlin, 1994*b*). This may indicate that synthesis or turnover of these proteins changes during the first few days of the last larval instar. Although glutamate dehydrogenase plays a central role in the metabolism of amino acids, the role of arginine kinase in the midgut has not been fully elucidated. This enzyme might be involved in regulating oxidative phosphorylation since two electrophoretically distinct isoenzymes are found in the mitochondria as well as the cytosol (Chamberlin *et al.* 1994). Whether the changes in arginine

kinase activity in the first 60 h of the fifth instar entail changes in isoenzyme composition or location remains to be determined.

The neural and hormonal factors that might control the changes in midgut metabolism and ion transport reported in this study have yet to be studied. On the basis of the results of the present study, as well as that of Sumner *et al.* (1995), ingestion of food does not appear to be an obligatory stimulus for the resumption of active ion transport after ecdysis. Therefore, activation of stretch receptors or chemoreceptors within the gut probably does not play a role in modulating the observed changes in midgut function. There are hormonal changes, however, which take place during the period when ion transport is suppressed and reinstated, and these hormonal changes may play a role in modulating midgut function before, during and after the molt. Prior to ecdysis, when midgut ion transport is depressed, ecdysteroid titers are high and juvenile hormone levels are falling (reviewed by Nijhout, 1994), and this hormonal environment might affect the function of both old and differentiating midgut cells. Ecdysis takes place when ecdysteroid levels are low and juvenile hormone levels are increasing (reviewed by Nijhout, 1994); after ecdysis, juvenile hormone titers remain high during the period in which the rate of ion transport is increasing. In addition to juvenile hormone and ecdysteroids, other hormones, such as eclosion hormone (reviewed by Nijhout, 1994) or ecdysone-triggering hormone (Zitnan *et al.* 1996), may modulate midgut function during the molting process. Whether these hormonal factors can be shown to affect midgut ion transport and metabolism *in vitro* remains to be seen.

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