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# CARBONIC ANHYDRASE ACTIVITY DURING THE LARVAL–PUPAL TRANSFORMATION OF *HYALOPHORA CECROPIA* REARED ON FOLIAGE AND SYNTHETIC DIET: EFFECTS OF POTASSIUM AND CHLORIDE ON MIDGUT, FAT BODY AND INTEGUMENTARY ENZYMES

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SUMMARY

1. Carbonic anhydrase was measured in tissues of silkmoths, *Hyalophora cecropia*, reared on either a wheatgerm-based synthetic diet or wild cherry foliage in feeding fifth-instar larvae, throughout the larval–pupal transformation and in newly ecdysed pupae.

2. Carbonic anhydrase activity was present in fat body, midgut and intgeumentary epithelial cells, but not in haemolymph, cuticle or the integumentary musculature.

3. Approximately 70% of the total carbonic anhydrase present per animal is localized within the epidermal cells of the integument.

4. The midgut is without measurable carbonic anhydrase activity from the day of apolysis (through the newly ecdysed pupal stage in development) until after the larval–pupal ecdysis.

5. In tissues analysed during the feeding fifth larval instar, I50 toward acetazolamide was between $10^{-8}$ and $10^{-7}$ M.

6. With the exception of larval midgut, regardless of stage, tissue or diet, both potassium and chloride normally *inhibit* carbonic anhydrase, with the effects of potassium and chloride being additive.

7. In larval midgut, chloride at concentrations of 50 or 150 mM *stimulates* carbonic anhydrase activity 30 or 100%, respectively.

8. In foliage-reared insects, potassium enhances chloride stimulation of larval midgut carbonic anhydrase, whereas in synthetic-diet-reared insects, potassium antagonizes the stimulatory effects of chloride.

9. Removal of a heat-stable, dialysable factor associated with the larval midgut of foliage-reared insects converts properties of the foliage midgut enzyme to those characteristic of the synthetic diet type, and vice versa.

## INTRODUCTION

Bicarbonate has been implicated in the production and movements of fluids in digestive and excretory systems of insects. The labial gland of *Antheraea pernyi* has been shown to secrete cocoonase in a potassium bicarbonate salt solution (Kafatos,

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1968). When tobacco hornworms (*Manduca sexta*) burrow underground at the onset of the larval–pupal transformation, they produce in either the midgut or the salivary gland an alkaline solution high in $K^+$ and $Na^+$ (Lockshin *et al.* 1978) and in bicarbonate (A. M. Jungreis, unpublished observations). Bicarbonate has been implicated in fluid and ion movements across the larval midgut of the Cecropia silkworm, *Hyalophora cecropia* (Haskell, Clemons & Harvey, 1965; Harvey, Haskell & Nedergaard, 1968), and the rectum of the locust, *Schistocerca gregaria* (Williams *et al.* 1978; Goh & Phillips, 1977). Bicarbonate not only plays a role in fluid movement in these systems, but also in the control of larval–pupal moulting fluid by the pharate pupal integument of lepidopteran insects. In *H. cecropia*, Jungreis & Harvey (1975) first proposed that bicarbonate is the counter ion passively transported with potassium during secretion of moulting fluid, and actively transported during resorption of moulting fluid (see Jungreis, 1979). A recent study of some of the anionic components in a haemolymph and moulting fluid in *H. cecropia* (Johnston & Jungreis, in preparation) revealed that bicarbonate was present in larval and pharate pupal haemolymph at concentrations as high as 35 mM, while that in moulting fluid was over 100 mM (Jungreis, 1979).

The origin of the bicarbonate in haemolymph is unknown. Haemolymph of both *H. cecropia* (Turbeck & Foder, 1970; Johnston, 1979) and *M. sexta* (Johnston, 1979) are reported to be without carbonic anhydrase activity. The source of the bicarbonate in moulting fluid is also unknown, since the quantity present in haemolymph of the late-feeding fifth instar is insufficient to account for the total present (haemolymph + moulting fluid) (Johnston, 1979) during elaboration of the larval–pupal moulting fluid.

Silkworms have been raised on both a wheatgerm-based synthetic diet (Riddiford, 1968) as well as wild cherry (*Prunus serotina*) foliage. The capacity and nature of alkali metal transport across the larval midgut of *H. cecropia* is influenced by diet (Blankemeyer & Harvey, 1977). Although differences in the concentrations of $K^+$ and $HCO_3^-$ in haemolymph of feeding larval stages are not present in silkworms reared on the two diets (Jungreis, Jatlow & Wyatt, 1973), substantial differences have arisen by the end of the larval–pupal transformation (Johnston, 1979; Jungreis, 1979). Haemolymph and moulting fluid pH of synthetic-diet-reared *H. cecropia* are similar to those of foliage-reared insects, yet the concentrations of $K^+$ and $HCO_3^-$ in moulting fluid of diet-reared insects are reduced by some 50 mM, while the osmolality of moulting fluid (relative to haemolymph) goes from hyperosmotic (Jungreis, 1974) to iso-osmotic (Jungreis, 1979). Thus, diet affects the composition of larval–pupal moulting fluid and, by inference, the regulation of carbonic anhydrase(s) present in the tissues.

In this report, carbonic anhydrase activities in midgut, fat body and integument of silkworms (*H. cecropia*) raised on both synthetic diet and wild cherry foliage were determined at a variety of developmental stages immediately before and throughout the larval-pupal transformation. Diet-specific total and diet-specific tissue carbonic anhydrase activities were determined by the electrometric method of Wilbur & Anderson (1948). The effects of $K^+$, $Cl^-$ and acetazolamide on these diet-specific tissue enzymes were then determined in an effort to gain insight into the regulation of diet- and tissue-specific carbonic anhydrases *in situ*.

## MATERIALS AND METHODS

### *Animal rearing*

Silkworms were reared in the laboratory on the artificial diet of Riddiford (1968) at 25 °C on a light regimen of 12 L:12 D. Animals were staged according to Jungreis (1979) and haemolymph and moulting fluid collected following procedures outlined in Jungreis (1974). Silkworms were also reared on wild black cherry foliage, *Prunus serotina*, in both the laboratory and in the field as described in Johnston & Jungreis (1980).

### *Carbonic anhydrase assay*

Carbonic anhydrase (CA) activity was assayed according to the electrometric method of Wilbur & Anderson (1948) as modified by Johnston & Jungreis (1979). Total and tissue-specific CA activity from individual animals was measured in the supernatant fraction of sonicated tissues following procedures described in Jungreis, Barron & Johnston (1980). The final concentration of $CO_2$ during measurement of carbonic anhydrase catalysed $CO_2$ hydration was normally 30·8 mM.

Tissues and fluids analysed for CA activity before, during and after the larval–pupal transformation were midgut, midgut contents, fat body, silk gland, integument, larval and pupal integumentary musculature, larval and pupal cuticle, larval and pupal integumentary epidermis, haemolymph and moulting fluid. Activity was detected only in fat body, midgut, midgut contents – of foliage- but not of synthetic-diet-reared silkworms – and epidermis (= integument). The effects of chloride as the choline salt, and $K^+$ as the $Cl^-$ salt were also studied in midgut, fat body and integument of foliage- and synthetic-diet-reared insects at the feeding larval and middle moulting stages in development. Due to its rather large size, choline ($Ch^+$) is assumed to be excluded from the catalytic site on carbonic anhydrase, and its substitution as a $Cl^-$ salt for alkali metal cations permits determination of an effect of $Cl^-$ in the 'absence' of cation (see Johnston & Jungreis, 1979). When the affects of ions on CA catalysed $CO_2$ hydration were measured, 0·1 Wilbur–Anderson (see Johnston & Jungreis, 1979) units of activity were initially present to facilitate comparisons between enzymes from different tissue sources. One W–A unit is defined as the quantity of enzyme needed to change the pH of 6 ml 15 mM HEPES buffer from pH 8·3 to pH 6·8 in 1 min.

### *Effects of acetazolamide*

Acetazolamide, a known inhibitor of mammalian (Maren, 1967) and insect carbonic anhydrases (Jungreis *et al.* 1980) was added to the final incubation mixture at (final) concentrations ranging from $10^{-10}$ to $10^{-5}$ M. The effects of this specific inhibitor were determined on midgut, midgut contents, fat body and integument of both foliage- and synthetic-diet-reared silkworms during the feeding fifth larval instar.

### *Dialysis of midgut carbonic anhydrase*

Foliage-reared *H. cecropia* larval midguts were prepared and sonicated according to Johnston & Jungreis (1979). Carbonic anhydrase activity in half of the supernatant fraction of the tissue sonicate (*ca.* 2·5 ml) was measured in duplicate in the presence

or absence of 50 mM KCl. The other half was dialysed against 50 ml 15 mM HEPES buffer (pH 8·35) and measured as described above. Dialysis was carried out with constant stirring at 3 °C in an Amicon Filtration chamber (Model 52) fitted with a PM 10 filter (which excludes particles > $10^4$ daltons) by applying 50 psi nitrogen pressure for 100 min (flow rate through the filter was 0·5 ml $min^{-1}$). Recovery of enzymic activity in the dialysed sample was 92%. No activity was detected in the filtrate. The enzyme was 'reconstituted' by adding 6 ml HEPES buffer filtrate to 0·5 ml of sample, and remeasuring the enzymic activity in the presence or absence of 50 mM KCl.

## RESULTS

### *Carbonic anhydrase activity in feeding larvae and throughout the larval–pupal transformation*

#### *Midgut*

Carbonic anhydrase activity in the midgut is highest at the feeding fifth stage in both foliage: and synthetic-diet-reared silkmoths [3·1 units $g^{-1}$ tissue weight or 2·1 total tissue units in foliage animals (Table 1); 2·8 units $g^{-1}$ or 1·9 total tissue units in synthetic-diet-reared animals (Table 2)]. After feeding ceases, but prior to initiation of spinning 12 h later, the animal purges its gut contents. Carbonic anhydrase activity in the gut is then found to fall precipitously (3·1 → 0·7, and 2·8 → 0·6 units $g^{-1}$, respectively; see Tables 1, 2). Three days after the initiation of spinning (apolysis), the gut is without measurable CA activity (Tables 1, 2). In fact, CA is not detected in midgut at any stage between apolysis and the newly ecdysed pupa under either rearing condition. It is unlikely that enzymic activity will return to the midgut even during the pupal–adult transformation, as *H. cecropia* does not feed as an adult (see Judy & Gilbert, 1969).

Virtually 100% of the CA activity in midgut is recovered from the cytoplasmic fraction of either sonicated tissues or those homogenized in glass or with a teflon pestle (see Jungreis *et al.* 1980). Interestingly, with foliage-reared insects only, extreme caution must be taken during the dissection to exclude from the excised tissue all leaf contamination within the gut contents. Up to 700 (range 400–700 units $g^{-1}$) units of carbonic anhydrase are present in the gut contents, which originate in *Prunus serotina* leaves. Plant CA is localized in a non-cytoplasmic fraction, and as little as 10 $\mu$g plant material per 500 mg gut will introduce a 50% error in the measured CA activity. Furthermore, plant CAs are *stimulated* by acetazolamide. Thus, it appears that activities and properties of *H. cecropia* midgut carbonic anhydrase reported by Turbeck & Foder (1970) are in error due to contamination with foliage from the midgut contents.

#### *Fat body*

The distribution of CA in fat body during the larval–pupal transformation (LPT) is very much dependent upon the rearing diet. In foliage-reared *H. cecropia*, fat body CA activity increases gradually throughout the LPT until the day of apolysis + 4 days, when a decline is initiated (Table 1). While initial increases can be assigned to putative transfers of enzyme from other tissues, later increases ($P < 0·05$) appear to arise

**Table 1.** *Carbonic anhydrase activities in selected tissues of foliage-reared silkmoths,* Hyalophora cecropia, *measured between feeding fifth larval and newly ecdysed pupal stages in development*

(Activity is expressed as the number of Wilbur–Anderson (W–A) units present per g wet weight of tissue and per animal, where insect weights were normalized at the end of the fifth instar to 15 g (see Jungreis & Tojo, 1973). Corrections for haemolymph present in blotted tissues were made after values listed in Jungreis & Tojo (1973). Total activity is the product of the haemolymph-corrected tissue wet weight per animal ($g^{-1}$) × carbonic anhydrase activity per g. In expressing carbonic anhydrase activity for integument, correction was made for the contribution of the cuticle to the integumentary weight. Values are the means ± standard errors for ($n$) individual insects.)

| | Midgut | | Fat body | | |
|---|---|---|---|---|---|
| Stage | W–A units $g^{-1}$ | W–A units animal$^{-1}$ | W–A units $g^{-1}$ | W–A units animal$^{-1}$ | Integument (W–A units $g^{-1}$) |
| Feeding fifth | 3·06 ± 0·488 | 2·10 ± 0·335 | 2·25 ± 0·386 | 2·03 ± 0·347 | 12·98 ± 0·477 |
| | $n = 15$ | $n = 15$ | $n = 15$ | $n = 15$ | $n = 15$ |
| Day of spin | 0·672 ± 0·078 | 0·329 ± 0·038 | 4·61 ± 0·569 | 5·07 ± 0·626 | 11·63 ± 0·986 |
| | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ |
| Spin + 1 day | 0·583 ± 0·065 | 0·255 ± 0·028 | 4·31 ± 0·359 | 4·74 ± 0·395 | 15·40 ± 1·64 |
| | $n = 4$ | $n = 4$ | $n = 4$ | $n = 4$ | $n = 4$ |
| Spin + 2 days | 0·188 ± 0·023 | 0·072 ± 0·009 | 4·30 ± 0·408 | 4·73 ± 0·449 | 17·67 ± 1·96 |
| | $n = 4$ | $n = 4$ | $n = 4$ | $n = 4$ | $n = 4$ |
| Apolysis | 0 | 0 | 7·27 ± 0·298 | 8·00 ± 0·373 | 20·27 ± 2·58 |
| | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ |
| Apolysis + 1 day | 0 | 0 | 6·24 ± 0·547 | 7·80 ± 0·766 | 24·55 ± 3·29 |
| | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ |
| Apolysis + 2 days | 0 | 0 | 7·43 ± 0·526 | 10·40 ± 0·815 | 23·43 ± 2·87 |
| | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ |
| Apolysis + 3 days | 0 | 0 | 6·65 ± 0·764 | 10·31 ± 1·31 | 20·71 ± 2·67 |
| | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ |
| Apolysis + 4 days | 0 | 0 | 6·71 ± 0·851 | 11·41 ± 1·45 | 18·86 ± 1·91 |
| | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ |
| Early moulting | 0 | 0 | 2·86 ± 0·248 | 5·29 ± 0·457 | 18·67 ± 1·77 |
| | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ |
| Middle moulting | 0 | 0 | 3·53 ± 0·106 | 6·53 ± 0·196 | 18·95 ± 1·84 |
| | $n = 8$ | $n = 8$ | $n = 8$ | $n = 8$ | $n = 8$ |
| Late moulting | 0 | 0 | 1·54 ± 0·038 | 2·85 ± 0·070 | 15·56 ± 2·60 |
| | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ |
| Pupae | 0 | 0 | 1·90 ± 0·099 | 3·80 ± 0·198 | 7·60 ± 0·563 |
| | $n = 5$ | $n = 5$ | $n = 5$ | $n = 5$ | $n = 5$ |

endogenously. Significant increases in total tissue activity occur between feeding larva and the day of spinning, spinning + 2 days and the day of apolysis, and apolysis + 1 day to apolysis + 2 days (Table 1). A 54% loss occurs between apolysis + 4 days and early moulting fluid (i.e. 48 h before the larval–pupal ecdysis). This loss then continues but at a reduced rate into the pupal stage of development.

In silkworms reared on synthetic diet, the pattern of CA activity during development is very different from that of foliage-reared animals. The increase in total CA activity observed in foliage-reared insects between the feeding larva and spinning + 2 days stages is absent, but a 5-fold (in contrast to a 2-fold) increase then occurs over the next 24 h between spinning + 2 days and apolysis (Table 2). This dramatic increase is followed just as rapidly by a drastic 64% loss over the next 24 h (apolysis to apolysis + 1 day). Like similarly staged foliage silkmoths, synthetic-diet-reared

Table 2. *Carbonic anhydrase activities in selected tissues of synthetic-diet-reared (l[redacted] silkmoths,* H. cecropia, *measured between the feeding fifth larval and newly ecdysed pupal stages in development. See Table* 1 *legend for details*

| | Midgut | | Fat body | | |
|---|---|---|---|---|---|
| Stage | W–A units $g^{-1}$ | W–A units animal$^{-1}$ | W–A units $g^{-1}$ | W–A units animal$^{-1}$ | Integument (W–A units $g^{-1}$) |
| Feeding fifth | 2·78 ± 0·879 | 1·91 ± 0·604 | 3·15 ± 0·447 | 2·84 ± 0·402 | 14·07 ± 1·43 |
| | $n = 6$ | $n = 6$ | $n = 6$ | $n = 6$ | $n = 6$ |
| Day of spin | 0·561 ± 0·271 | 0·274 ± 0·133 | 1·93 ± 0·095 | 2·12 ± 0·105 | 10·18 ± 2·06 |
| | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ |
| Spin + 1 day | 0·501 ± 0·222 | 0·220 ± 0·097 | 2·53 ± 0·342 | 2·78 ± 0·376 | 13·27 ± 1·68 |
| | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ |
| Spin + 2 days | 0·283 ± 0·186 | 0·109 ± 0·072 | 1·54 ± 0·189 | 1·69 ± 0·208 | 16·74 ± 1·44 |
| | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ |
| Apolysis | 0 | 0 | 7·64 ± 1·10 | 8·40 ± 1·21 | 35·20 ± 2·64 |
| | $n = 6$ | $n = 6$ | $n = 6$ | $n = 6$ | $n = 6$ |
| Apolysis + 1 day | 0 | 0 | 2·40 ± 0·323 | 3·00 ± 0·404 | 11·86 ± 0·860 |
| | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ |
| Apolysis + 2 days | 0 | 0 | 4·79 ± 0·765 | 6·71 ± 1·07 | 13·57 ± 1·95 |
| | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ |
| Apolysis + 3 days | 0 | 0 | 4·06 ± 0·452 | 6·29 ± 0·701 | 10·43 ± 1·73 |
| | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ |
| Apolysis + 4 days | 0 | 0 | 4·00 ± 0·556 | 6·80 ± 0·945 | 10·29 ± 1·72 |
| | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ |
| Early moulting | 0 | 0 | 9·73 ± 1·85 | 18·00 ± 3·42 | 30·00 ± 2·89 |
| | $n = 3$ | $n = 3$ | $n = 5$ | $n = 5$ | $n = 5$ |
| Middle moulting | 0 | 0 | 8·95 ± 1·97 | 16·56 ± 3·64 | 13·33 ± 1·76 |
| | $n = 8$ | $n = 8$ | $n = 8$ | $n = 8$ | $n = 8$ |
| Late moulting | 0 | 0 | 8·34 ± 1·95 | 15·43 ± 3·60 | 10·93 ± 0·958 |
| | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ |
| Pupae | 0 | 0 | 10·10 ± 2·03 | 20·20 ± 4·06 | 9·60 ± 1·43 |
| | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ | $n = 3$ |

insects have invariant but lower levels of CA activity between apolysis + 2 days to apolysis + 4 days. Synthetic-diet-reared silkworms have radically different patterns of CA activity from apolysis + 4 days until the larval–pupal ecdysis. Carbonic anhydrase activity triples between apolysis + 4 days and early moulting fluid (a 24 h period), whereas it declined 50% in foliage animals (contrast Tables 1 and 2), only to be followed by a slight decline in total activity between early and late moulting fluid (18 versus 15 units). As the process of moulting fluid resorption terminates and the animal prepares to moult, levels of CA increase to over 20 units. Thus synthetic-diet-reared silkworms enter the pupal stage with 20 units of CA in fat body, while foliage-reared insects have only 4.

*Integument*

As was observed with fat body, CA activity in integument is very much dependent upon diet. Total integumentary CA in foliage-reared *H. cecropia* declines sharply between the fifth instar and initiation of spinning (Fig. 1). Carbonic anhydrase activity in integument then increases slightly following initiation of spinning but prior to completion of the outer cocoon (12 h), but becomes stable thereafter for 2 days

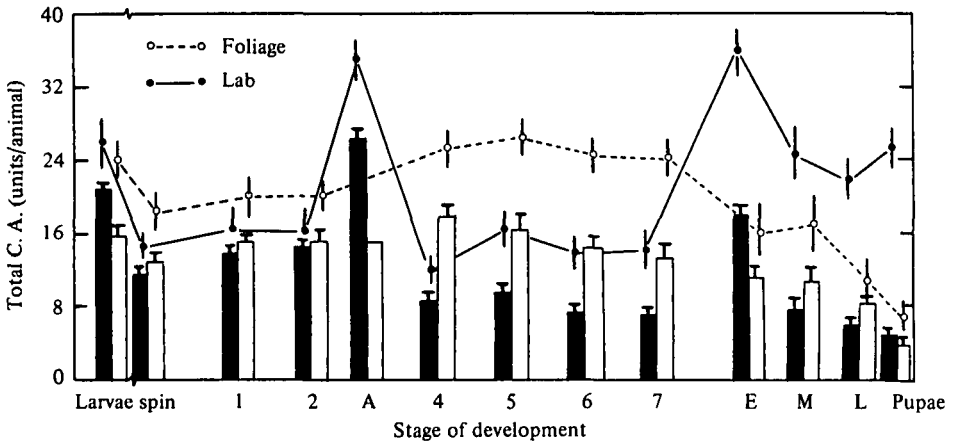


Fig. 1. Total carbonic anhydrase in silkmoths, *H. cecropia*, reared on either synthetic diet (lab) or foliage, and measured at the end of the feeding fifth instar, throughout the larval–pupal transformation, and in newly ecdysed pupae. Activity is expressed as total Wilbur–Anderson (W–A) units per animal and per integument at each stage in development. A, day of apolysis; spin, day of spinning; E, early moulting fluid; M, middle moulting fluid; L, late moulting fluid. Values are the means ± standard errors for $n$ individuals (see Tables 1 and 3 for the numbers of individuals employed). Closed circles/solid lines, total activity in synthetic-diet-reared insects; open circles/dashed lines, total activity in foliage-reared insects; solid bars represent enzyme activity in integument of synthetic-diet-reared insects; open bars represent activity in integument of foliage-reared insects.

(spin + 1 day through apolysis). A second increase in activity occurs between apolysis and apolysis + 1 day, whereupon levels decline only gradually through the early moulting stage (a total loss of 10%) with accelerated losses occurring between middle moulting and the newly ecdysed pupa (Fig. 1).

In synthetic-diet-reared insects, total CA in integument is the same during the first three days of the larval–pupal transformation (Fig. 1). Thereafter, significant ($P < 0{\cdot}05$) differences attributed to diet are noted at each successive stage in development up to, but not including, the newly ecdysed pupa (Fig. 1). As in foliage-reared insects, there is a sharp decline in CA activity in the integument between the fifth instar and initiation of spinning. Levels of CA change little between spinning and spinning + 2 days, although foliage-reared insects had 25% more activity (Fig. 1). Apolysis triggers diet-specific changes in total activity. Synthetic-diet-reared insects experience a 2-fold increase in CA activity on the day of apolysis, followed one day later by a 60% loss such that foliage insects now have both twice the total and twice the integumentary activity of diet-reared insects (Fig. 1, Tables 1, 2). Activity in silkworms on both diets then remains stable through apolysis + 4 days, when integuments from synthetic-diet- but not foliage-reared insects exhibit a 150% increase in activity (14 → 36 units), which declines just as dramatically as it rose until only 5 units remain in new pupae. Apart from the tremendous increase in activity noted in early moulting animals, foliage-reared insects also experience a dramatic 70% loss between apolysis + 1 day and the larval–pupal ecdysis (Fig. 1).

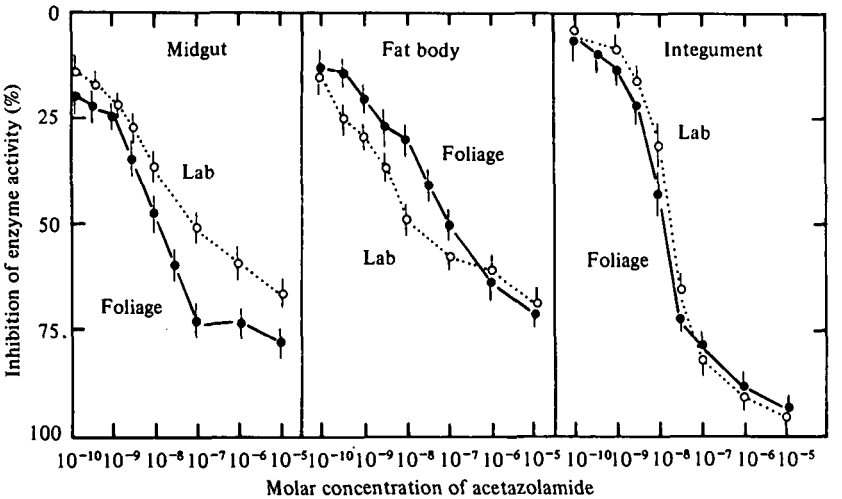


Fig. 2. Effects of acetazolamide on tissue-specific carbonic anhydrase activities in both synthetic-diet-reared (lab) and foliage-reared silkmoths, *Hyalophora cecropia*, during the late-feeding fifth larval instar. Activity is measured in midgut, fat body and integument both in the absence (0% inhibition) and presence of $10^{-10}$–$10^{-5}$ M acetazolamide (final concentrations). Each point represents the mean (±standard error) of at least three separate animals each run at least in duplicate.

*Effects of acetazolamide on tissue- and diet-specific carbonic anhydrases*

Acetazolamide is more effective in inhibiting midgut carbonic anhydrase in foliage-reared animals than in those fed on synthetic diet ($I_{50}$ $10^{-8}$ and $10^{-7}$ M, respectively, Fig. 2). Acetazolamide does not inhibit but activates (20% at $10^{-8}$ M) CA present in gut contents of foliage animals. This observation is extremely useful in differentiating between nascent midgut enzyme and contaminating plant enzyme. Carbonic anhydrase in midgut is not unique in possessing a diet-specific $I_{50}$ towards acetazolamide, since the values of $I_{50}$ for foliage- versus synthetic-diet-reared fat body enzymes are $10^{-7}$ and $10^{-8}$ M, respectively. Integumentary carbonic anhydrases have the same $I_{50}$ regardless of diet ($3 \times 10^{-8}$ M); this value is intermediate between those of midgut and fat body. Similarity of $I_{50}$ in foliage- versus diet-reared insects should not be interpreted as evidence that the same enzyme is present in both tissues (see below).

*Effects of $K^+$ and $Cl^-$ on tissue- and diet-specific carbonic anhydrases*

*Midgut*

Addition of KCl at concentrations between 5 and 50 mM (at a $CO_2$ concentration of 30·8 mM) causes a linear *activation* of the midgut enzyme of the foliage animals but not the synthetic diet animals; in the latter, the enzyme is *inhibited* some 50% by 50 mM-KCl (Fig. 3). This KCl activation in foliage animals is an effect of chloride (70–80% of the activation based on the response of the enzyme in choline chloride), which in mammals (Johnston & Jungreis, 1979) and *M. sexta* (Jungreis *et al.* 1980) causes an inhibition of CA activity. The inhibition exhibited by diet-reared enzyme is caused by $K^+$ (see the line for ChCl in Fig. 3), an observation consistent with the response of many mammalian enzymes (Johnston & Jungreis, 1979).

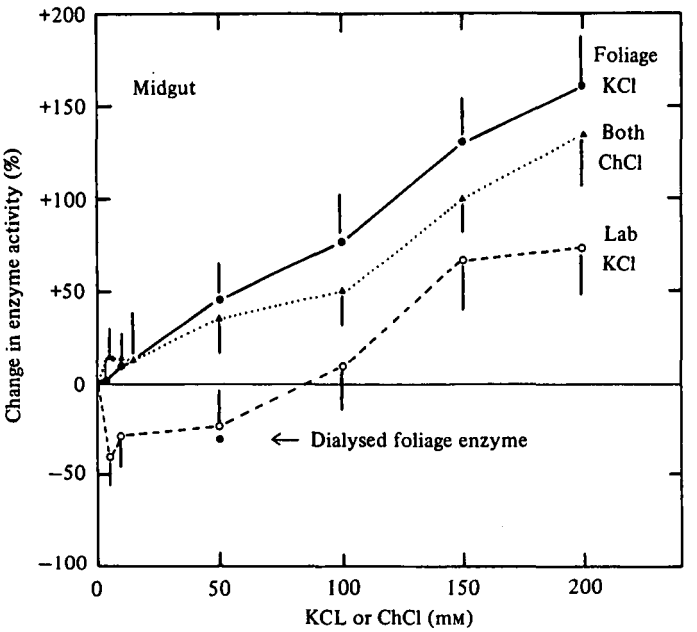


Fig. 3. Effects of chloride (as choline chloride) and potassium (as potassium chloride) on midgut carbonic anhydrase activity from synthetic-diet (lab)- and foliage-reared silkmoths, *H. cecropia*, during the feeding fifth instar. The line marked 'both' ChCl indicates that the effect of ChCl on the foliage enzyme is not significantly different from the response of the synthetic-diet-reared enzyme. The point labelled dialysed foliage enzyme represents the effect of 50 mM KCl on foliage midgut carbonic anhydrase following dialysis (see text). Each point represents the mean (± standard errors) of at least three separate animals each run at least in duplicate.

In midgut, differential diet-dependent responses to $K^+$ and $Cl^-$ suggested that an allosteric effector contributed by the diet might be present in one or both of the enzyme preparations. When supernatant fractions from foliage midgut sonicates were dialysed against 15 mM HEPES buffer, the enzyme was transformed from the foliage to the synthetic diet type (Fig. 3), that is, the enzyme previously *activated* 50% by 50 mM KCl was now *inhibited* 40 ± 10% ($n = 3$). Reconstitution of the foliage enzyme was possible by addition of dialysate to dialysed enzyme. Filtration of the synthetic-diet-type enzyme did not influence the effects of KCl on its CA activity. More importantly, sonication of the synthetic diet enzyme in the foliage dialysate converted it to the foliage type (30 ± 11% activation, $n = 3$, in contrast to the normal 40% inhibition). In two experiments, dialysate from foliage midguts was heated in a boiling water bath for 10 min. Boiled dialysate was still able to restore the characteristic foliage activity profile to the dialysed enzyme, and to convert the synthetic diet enzyme to the foliage type. Thus, foliage- and synthetic-diet-reared midgut CAs can be interconverted by the addition or removal of a non-labile dialysable component present only in foliage insects. Interestingly, at concentrations above 50 mM, the effects of $K^+$ plus $Cl^-$ on the two enzymes are identical, with activation profiles for enzymes from animals reared on the two diets having identical slopes (Fig. 3).

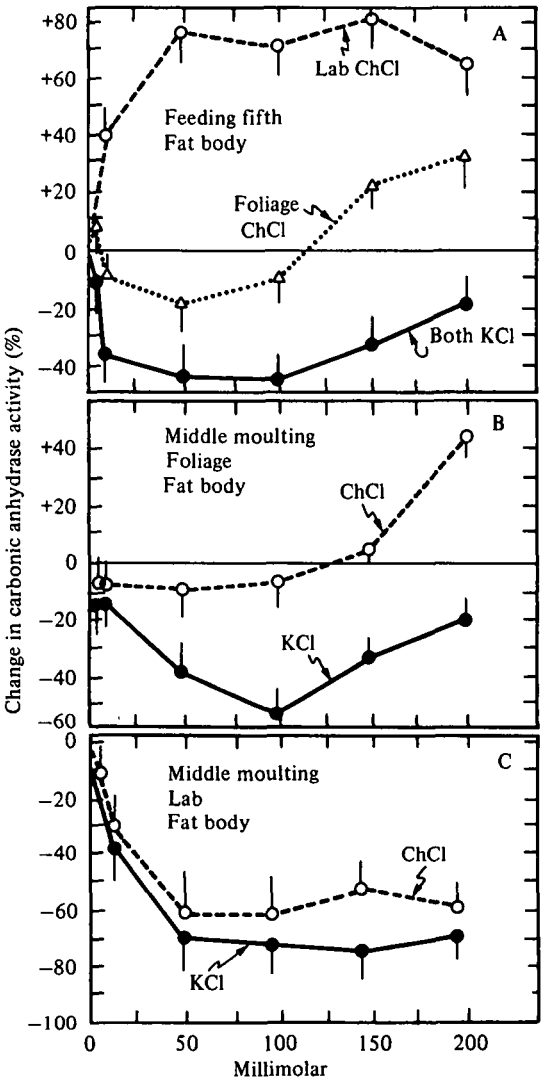


Fig. 4. Effects of chloride and potassium on fat body carbonic anhydrase activity from synthetic-diet(lab)- and foliage-reared silkmoths, *H. cecropia*. (*a*) Feeding fifth instar – the line marked 'both' KCl indicates that the effects of KCl on enzymes from the two diets were not significantly different; (*b*) middle moulting pharate pupae – foliage reared; (*c*) middle moulting pharate pupae – synthetic diet reared.

*Fat body*

The effects of $K^+(Cl^-)$ and $(Ch^+)Cl^-$ on fat body CA in both fifth-instar foliage- and synthetic-diet-reared *H. cecropia* are shown in Fig. 4*a*. Enzymes from both foliage- and diet-reared tissues are inhibited 40% at KCl concentrations between 10 and 100 mM. In foliage animals, this inhibition is due to the additive effects of $K^+$ and $Cl^-$ (see line marked foliage ChCl in Fig. 4*a*), while in synthetic-diet-reared insects, since $Cl^-$ greatly stimulates (80%) fat body CA, inhibition seen in the presence of KCl is due entirely to $K^+$ (see line marked lab ChCl in Fig. 4*a*).

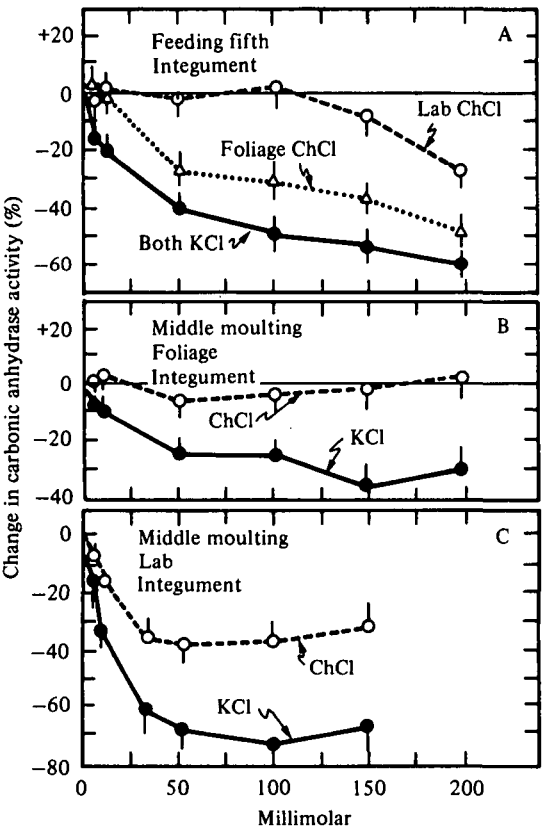


Fig. 5. Effects of chloride and potassium on integumentary carbonic anhydrase from synthetic-diet(lab)- and foliage-reared silkmoths, *H. cecropia*. (*a*) Feeding fifth instar – the line marked 'both' KCl indicates that the effects of KCl on enzymes from the two diets were not significantly different; (*b*) middle moulting pharate pupae – foliage reared; (*c*) middle moulting pharate pupae – synthetic diet reared.

The effect of KCl at concentrations up to 200 mM were also determined with fat body CA from foliage animals at the middle moulting fluid stage (Fig. 4*b*). These animals produce moulting fluid whose $K^+$ concentration is 160 mM (Jungreis, 1974) and whose integumentary $K^+$ levels are at least 175 mM (Jungreis & Tojo, 1973). KCl inhibits 20–40% at concentrations between 10 and 200 mM, with 100 mM KCl providing the greatest inhibitory effect (40%). Inhibition below 100 mM is an additive effect of $K^+$ and $Cl^-$, and above 100 mM it is due solely to $K^+$. The effects of $K^+$ and $Cl^-$ do not differ significantly from those seen with enzyme from feeding fifth instars (compare Fig. 4*a* and *b*).

The effects of KCl on CA from synthetic-diet-reared insects change dramatically between the fifth instar and middle moulting fluid stages (compare Fig. 4*a* and *c*). Chloride which had activated the enzyme 80% in the fifth instar now inhibits the enzyme 60% during the middle moulting fluid stage (Fig. 4*c*).

*Integument*

Integument carbonic anhydrases from foliage- and diet-reared animals are inhibited some 50–60% by KCl concentrations between 40 and 100 mM (Fig. 5*a*). In foliage animals, this inhibition is due to the combined effect of $K^+$ and $Cl^-$ (see foliage ChCl line, Fig. 5*a*), while in synthetic-diet-reared animals it is due entirely to $K^+$ (i.e. $Cl^-$ has no effect on the enzymic activity; see lab ChCl line in Fig. 5*a*). During the middle moulting stage, chloride no longer inhibits foliage CA (Fig. 5*b*), which is now inhibited 20–30% primarily by $K^+$ at concentrations of 25–200 mM. In synthetic-diet-reared silkworms, integumentary carbonic anhydrase changes between the fifth instar and the middle moulting fluid stage such that $Cl^-$, which was without effect in the feeding larva, now becomes inhibitory (40%, Fig. 5*c*).

## DISCUSSION

The present study has shown that neither haemolymph, muscle, cuticle, silk gland nor moulting fluid contains CA activity. Plant CA was found to be a contaminant of foliage- but not synthetic-diet-reared midgut CA, and all of the properties ascribed to *H. cecropia* midgut CA by Turbeck & Foder (1970) can, in fact, be attributed to the contaminating *P. serotina* plant CA. Since the concentration of $CO_2$ greatly influences the extent of cation or anion activation or inhibition of the various insect CAs (Johnston, 1979; Johnston & Jungreis, 1979), the concentration of $CO_2$ was maintained at around 30·8 mM throughout, which is physiological in this species (Johnston, 1979).

*Midgut*

Studies on the effects of acetazolamide, $K^+$ and $Cl^-$ demonstrate that the responsiveness of midgut CA is very much dependent upon diet. The ease of interconverting diet-specific enzymes following dialysis indicates that the diet of synthetic-diet-reared silkworms is lacking an as yet undetermined factor or factors, which cause $K^+$ to inhibit rather than activate the midgut enzyme (Fig. 3). This situation is analogous to that described in *H. cecropia* by Blankemeyer (1976; Blankemeyer & Harvey, 1977), where electrical coupling of goblet with columnar cells occurs only in midguts from synthetic-diet-reared insects. It thus appears that different rearing techniques can be used to answer questions about enzyme production and regulation, as well as trans-cellular transport of ions. Whether the factor responsible for transformation of midgut CA is the same one responsible for electrical coupling is unknown.

High levels of CA seen in midgut tissue from feeding fifth animals are probably involved in the electrogenic transport of $K^+$ by the gut (Harvey & Nedergaard, 1964) or of activation of $HCO_3^-$-specific ATPases (Deaton & Jungreis, 1980). In the stomach of vertebrates, CA is involved in the secretion of $H^+$, and an analogous situation of bicarbonate transport into midgut lumen is not unlikely. Ethoxazolamide (a potent inhibitor of carbonic anhydrase) has been shown to inhibit the short-circuit current of the foliage integument by 36%, while sodium sulphide (a non-competitive inhibitor of CA) completely inhibited the current generated by the silkmoth midgut

Haskell *et al.* 1965). The short-circuit current measured in the isolated rectum of the desert locust *Schistocerca gregaria* has within it a substantial contribution by actively transported $HCO_3^-$, and it is also inhibited by acetazolamide (Williams *et al.* 1978).

The presence of an $HCO_3^-$-stimulated ATPase has been demonstrated in the larval midgut of *H. cecropia* (Turbeck, Nedergaard & Kruse, 1968) and *Manduca sexta* (Deaton & Jungreis, in preparation). Activation of enzymes by $HCO_3^-$ is not a unique property assigned only to insect enzymes, since $HCO_3^-$ activation has been reported for several unlikely enzymes such as phospho-enolpyruvate carboxykinase ferro-activator (Merryfield, 1979) and a cyclic-AMP-dependent transport mechanism in frog erythrocytes (Rudolph, 1979).

*Fat body*

An increase in fat body CA is first noted in foliage animals between the fifth instar and the initiation of spinning (Table 1). It results from an approximate doubling of CA specific activity, since the total tissue mass changes only slightly from 0·9 to 1·1 g (cf. Table 1). A second increase noted between spinning + 2 days and the day of apolysis is also due to a 1·7-fold enhancement of the specific activity (Table 1), since total fat body weight is the same at both stages. However, the third increase (27%) in total activity occurring between apolysis + 1 day and apolysis + 2 days is due to a combination of increasing fat body tissue weight (16%) and increased specific activity of the enzyme (11%; see Table 1). Thereafter, from apolysis + 2 days until the larval–pupal ecdysis, despite a 30% *increase* in fat body weight, total CA in foliage fat body *declines* 75% due either to an absolute loss of enzyme, or to a masking of the nascent CA enzyme capacity (Fig. 1).

Enzymic activity in fat body from foliage- and synthetic-diet-reared insects is most different between apolysis + 4 days and the larval–pupal ecdysis, a period when CA activity in fat body of synthetic-diet-reared insects more than triples, in contrast to a 67% decline in fat body of foliage-reared insects. In this report we noted that the two diet-associated fat body CAs are differentially affected by $Cl^-$ (Fig. 4*a*, *c*) This seeming differential CA responsiveness to $Cl^-$ *in vitro* may be part of the normal *in vivo* regulatory process. Fat body CA from synthetic diet insects is inhibited by $Cl^-$. Since haemolymph $Cl^-$ levels are elevated in synthetic-diet- versus foliage-reared insects (Jungreis *et al.* 1973), fat cells from synthetic-diet insects could contain significantly higher levels of intracellular $Cl^-$ than cells from foliage-reared animals. One might then anticipate a functional reduction in the total *in situ* CA activity in synthetic-diet fat body due to its elevated $Cl^-$. An incorrect picture of CA activity is thus obtained when the enzyme is measured *in vitro* due to the absence of this $Cl^-$. Supporting this proposed role of $Cl^-$ *in situ* in fat body of synthetic-diet-reared insects is the observation that, during elaboration of moulting fluid, foliage-reared *H. cecropia* have 3–5 times more $HCO_3^-$ in haemolymph than do synthetic-diet-reared insects (Johnson, 1979; Jungreis, 1979). Let us assume that CA activity in fat body is responsible for the production the $HCO_3^-$ that appears in haemolymph, an assumption validated by the observed lack of CA activity in tissues other than integument. In fat body of synthetic diet *H. cecropia*, CA is so inhibited by $Cl^-$ *in vivo*

that haemolymph $HCO_3^-$ levels are only modest (5 mM in synthetic diet versus 30+ mM in foliage insects). The CA enzymic capacity cannot be expressed *in vivo* in the presence of high intracellular $Cl^-$. When synthetic-diet fat body CA is removed from its inhibitory $Cl^-$ environment during assay *in vitro*, a tremendous increase in CA activity is recorded spuriously.

This anomaly is not encountered by foliage animals for two reasons. First, $Cl^-$ does not affect the enzyme at the moulting fluid stage in development (Fig. 4*b*). Secondly, intracellular $Cl^-$ levels are probably low relative to those in animals reared on synthetic diet. Thus foliage-reared silkworms are capable of producing higher levels of $HCO_3^-$ in haemolymph than diet-reared insects, despite similar levels of CA activity measured under *in vitro* conditions.

*Integument*

The decline in measured integumentary CA between the feeding larva and the day of spinning in insects reared on either diet (Fig. 1) is the product of decreases in integumentary weight (27%) and in enzymic activity (10–25%). Despite a continuous drop in integumentary weight between the days of spinning and apolysis, total CA activity remains stable in both rearing groups. At, and only at, apolysis there is an inexplicable rise in the CA activity in synthetic-diet-reared insects only (Fig. 1). A second transitory diet-specific increase in CA activity also occurs in synthetic-diet insects at the early moulting stage (Fig. 1). Carbonic anhydrase activity in integument declines in insects reared on both diets between early moulting and the larval–pupal ecdysis.

The differential effects of $Cl^-$ on integumentary CA noted at the feeding fifth stage (Fig. 5*a*) and the differential effects of both $K^+$ and $Cl^-$ at the moulting fluid stage (contrast Figs. 5*b* and *c*) reveals the presence of fundamental differences between the diet-specific integumentary enzymes.

In an analysis of *H. cecropia* haemolymph and moulting fluid, bicarbonate levels in moulting fluid of synthetic-diet-reared insects were only half of those in foliage-reared animals (Johnston, 1979; Jungreis, 1979). Differences in the regulation of the diet-specific CAs are responsible for the observed differences in the composition of moulting fluid (Jungreis, 1974, 1979). At the time of moulting fluid elaboration, CA measured in integument of synthetic-diet-reared insects is sufficient to produce $HCO_3^-$ at rates comparable to those measured in the integument of foliage-reared insects. However, the synthetic diet enzyme is twice as sensitive to $K^+$ and $Cl^-$ as is the foliage enzyme (contrast Figs. 5*b* and *c*). We propose that the cation and anion inhibition of integumentary CA normally masks the CA capacity *in situ*, with the result that synthetic diet integument can produce only half of the moulting fluid $HCO_3^-$ equivalents that are measured in moulting fluid of foliage-reared insects (Johnston, 1979; Jungreis, 1979).

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