To the Graduate Council:

I am submitting herewith a dissertation written by James Wilson Johnston entitled "Carbonic Anhydrase and Bicarbonate Regulation During the Larval-Pupal Transformation of the Tobacco Hornworm (Manduca sexta) and the Silkworm (Hyalophora cecropia)." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Zoology.

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B

CARBONIC ANHYDRASE AND BICARBONATE REGULATION DURING THE LARVAL-PUPAL TRANSFORMATION OF THE TOBACCO HORNWORM (*Manduca sexta*) AND THE SILKWORM (*Hyalophora cecropia*)

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

James Wilson Johnston

December 1979

ACKNOWLEDGMENTS

The author is grateful for the assistance, guidance and patience of Dr. Arthur M. Jungreis, and the aid and support of the members of the dissertation committee: Dr. M. L. Pan, Dr. J. N. Liles, Dr. R. H. Feinberg and Dr. J. T. Blankemeyer.

Recognition is given also to the following persons who have assisted with carrying out many of the experiments and clarifying portions of this manuscript: J. Michael Henry; Christine Whitnable; Harold Glass, Jr.; and Mila Arceneaux.

I would like to thank Patsy Boyce for making portions of her masters thesis available to me, and Dr. Gary Whitson for providing some of the isotopic material used in these experiments.

I must remember likewise the invaluable assistance of Paul and Sue Lynch in helping me prepare the final draft of this manuscript.

Finally, I would like to offer a special thanks to my wife, Karen, for her endless support and encouragement during the entirety of my graduate career.

ABSTRACT

This study represents an effort to answer basic questions concerning physiological mechanisms involved in the larval-pupal transformation of lepidopterous insects. More specifically it examines the role of bicarbonate in the process of moulting in the tobacco hornworm, Manduca sexta, and the silkmoth, Hyalophora cecropia. This role has been evaluated through an examination of:

1. the levels of bicarbonate in hemolymph and moulting fluid,
2. the mode and sites of bicarbonate production,
3. the regulation of this production, and
4. the physiological mechanisms of bicarbonate involvement in the secretion and resorption of moulting fluid.

In order to effectively quantitate levels of bicarbonate in the extremely small fluid volumes associated with insects, a new technique for assaying carbon dioxide under conditions of small fluid volumes has been devised. By this technique it is now possible to cheaply and conveniently assay micromolar quantities of carbon dioxide in fluid volumes as small as ten microliters.

The mode and sites of bicarbonate production were evaluated by assaying for the major enzyme involved, carbonic anhydrase. This study represents the first time that carbonic anhydrase has been reported in insect fat body and integument. In addition, important questions dealing with the regulatory effects of halide anions and alkali metal cations on carbonic anhydrase activity in a variety of insect tissues

have been answered. In M. sexta all three enzymes (midgut, fat body and integument) are activated by physiological levels of K^+ and either inhibited or unaffected by physiological levels of Cl^-. In foliage reared H. cecropia both K^+ and Cl^- are inhibitory to the three tissue enzymes, while lab reared feeding fifth midgut carbonic anhydrase is activated by Cl^-.

These regulatory effects differ from the anion and cation effects on mammalian carbonic anhydrase. In fact, Cl^- is reported to always be inhibitory to mammalian carbonic anhydrase, while the effects of cations have not been reported. In addition, a major controversy has arisen regarding the kinetics of this Cl^- inhibition. Cl^- inhibition is reported to be noncompetitive during CO_2 hydration and competitive during H_2CO_3 dehydration. By employing proper controls and by analogy to the insect enzyme (especially in M. sexta, in which K^+ activates and Cl^- inhibits), the controversy regarding the inhibitory mechanism of Cl^- on mammalian carbonic anhydrase has been resolved. The kinetics of Cl^- inhibition are different because of the presence (noncompetive) or absence (competitive) of K^+.

Finally, through the use of radioisotopic flux determinations and tissue voltage clamping techniques, the physiological involvement of bicarbonate movement in the processes of secretion and resorption of moulting fluid has been ascertained. Bicarbonate accounts for electroneutrality, pH optium and osmotic equivalency during moulting fluid secretion, while the active movement of bicarbonate back into hemolymph from the exuvial side during resorption of moulting fluid provides the driving force for this resorptive process.

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INTRODUCTION

The tobacco hornworm, Manduca sexta, and the silkmoth, Hyalophora cecropia, are two of the most extensively studied and widely used insect models. They have been extensively used in the study of insect endocrinology, morphologic development, control of protein and nucleic acid synthesis et cetera. Of particular interest is the use of the two insects in studies dealing with moulting, that is, the periodic shedding of the old cuticle (ecdysis) in order to grow and to develop. At the onset of each moult cycle a characteristic sequence of events is initiated which results both in the formation of a new cuticle and in the digestion and shedding of the old cuticle (see Jungreis, 1979, for an extensive review of the literature). The partial freeing of the old cuticle from the underlying epidermis has been given the term apolysis. Following upon the heels of apolysis is the formation of a moulting or exuvial space. The exuvial space is a region bounded by the newly synthesized ecdysial membrane and the endocuticular portion of the "old" cuticle. Ecdysis and moulting are always preceeded by the introduction of moulting fluid into the ecdysial space with its accompanying processes of cuticle digestion and hydrolysate resorption. The nature of moulting fluid, its mechanism of formation and the role of HCO_3^- and integumentary carbonic anhydrase in the elaboration and resorption of this fluid during the larval-pupal transformation of Hyalophora cecropia and Manduca sexta are described in this study.

Previous studies on the transepithelial movement of fluid and ions during the larval-pupal transformation have dealt primarily with the active transport of K^+ across the midgut from hemolymph toward the gut lumen as well as the transport of K^+ across the integument from hemolymph toward the exuvial (moulting fluid) space. In conjunction with these studies, the composition of hemolymph, moulting fluid and gut contents have been analyzed in these two species. These analyses have dealt primarily with changes in K^+, Na^+, Mg^{++} and Ca^{++}, with Cl^- being the only anion categorized. Initial studies of ion transport by the integument failed to reveal the anion accompanying K^+. The present study represents an attempt to gain a more complete picture of the anionic composition of hemolymph and moulting fluid in order to pinpoint that anion. Bicarbonate has been postulated as the anion accompanying actively transported K^+ across the integumentary epithelium during the formation of larval-pupal moulting fluid. An analysis of the bicarbonate levels in hemolymph and moulting fluid throughout this entire process was therefore undertaken.

Bicarbonate has not only been implicated in the process of moulting fluid secretion and resorption, but in labial gland secretions and rectal resorption of water as well. However, no study of the change in bicarbonate levels in hemolymph over an extended portion of insect development has been undertaken. In addition, the bicarbonate concentration in insect moulting fluid has never been determined. It is apparent that a complete picture of the above mentioned physiological systems in insects is not possible until bicarbonate levels and the mode, site and regulation of bicarbonate production are fully

investigated. Since K^+ transport across the midgut of M. sexta and H. cecropia has been studied exhaustively, both species are excellent candidates for a study of bicarbonate involvement in these processes.

With the advent of artificial insect diets it became possible to raise both M. sexta and H. cecropia on synthetic diets. Wild cherry foliage of H. cecropia is more readily available than is the natural tobacco foliage of M. sexta. Thus, silkmoths are commonly reared by both techniques, while tobacco hornworms are rarely raised on their natural diet. Bicarbonate levels were therefore analyzed under both rearing conditions (artificial diet and natural foliage) in H. cecropia, but only under synthetic diet conditions in M. sexta.

The mode of production of bicarbonate, present in hemolymph and moulting fluid, was determined by assaying for the enzyme carbonic anhydrase. Since carbonic anhydrase catalyzes the reversible hydration of CO_2 to form carbonic acid (H_2CO_3), it is the major enzyme involved in the production of bicarbonate. However, no study to date has characterized carbonic anhydrase activity in any insect tissue over an extended developmental period. In fact, previous reports on insect carbonic anhydrases have dealt primarily with the lack of the enzyme in hemolymph at only one developmental stage. Although carbonic anhydrase is indeed absent from the hemolymph of both M. sexta and H. cecropia, it is present in large quantities in midgut, fat body and integument of both insects. This study is the first report of the presence of carbonic anhydrase in fat body and integument.

In order to carry the study of bicarbonate's role in the larval-pupal transformation one step further, the effects of alkali

metal cations and halide anions on carbonic anhydrase activity have been determined. This was done in an effort to gain insight into some of the factors involved in regulation of bicarbonate production. By assaying the enzyme *in vitro* in the presence of physiological concentrations of K^+ and Cl^-, *in vivo* conditions are more closely approximately and the regulatory effects of major ions observed. The results of these studies proved so extraordinary that major controversies regarding the kinetics of inhibition of mammalian carbonic anhydrases were resolved after gaining insight into the insect exzymes.

Finally, the role of bicarbonate in the larval-pupal transformation was evaluated via the use of radioisotopic flux measurements and tissue voltage clamping techniques on isolated integuments from the tobacco hornworm, *M. sexta*. These studies revealed that bicarbonate is indeed the missing anion involved in K^+ transport across the integument. Further, it appears that active transport of bicarbonate from exuvial space to hemolymph during resorption of moulting fluid may be the driving force during the resorptive process.

In summary, this is a study of bicarbonate and its role in insect moulting. The manner in which lepidopterous insects produce bicarbonate is uncovered, as well as the relative importance of major tissues (midgut, fat body and integument) in that production. Further, this study evaluates the regulatory role played by important anions (Cl^-) and cations (K^+) in the stage specific inhibition and activation of carbonic anhydrase. Finally, this study traces the mechanisms by which bicarbonate movement allows the insect to grow and develop.

CHAPTER 1

A SIMPLIFIED METHOD FOR ASSAYING MICROMOLAR QUANTITIES OF CO_2

I. INTRODUCTION

A knowledge of the concentration of bicarbonate (HCO_3^-) in biological fluids is often necessary if mechanisms for effecting homeostasis are to be understood. A variety of methods have been developed over the past 40 years for the analysis of HCO_3^- in biological fluids. These methods are of three types: (1) inexpensive and sensitive, but extremely tedious (Little and Ruston, 1969); (2) routine methods requiring large sample sizes (0.2-1.0 ml) and of limited resolution (1.2-4.5 microequivalents) (Van Slyke and Neill, 1924; Severinghaus and Bradley, 1958; Snell, 1960; Conway, 1962); or (3) specialized methods of high sensitivity (10^{-11} moles) requiring high equipment costs ($12,000-$40,000) and long set-up times (hours) (Prop, 1954; Van Bruggen and Scott, 1962; Maffly, 1968; Pita, 1969; Williams, Woods and Umstead, 1972; Hevert, 1973; Karlmark and Sohtell, 1973; Caflish and Carter, 1974; and Vurek, Warnock and Corsey, 1975), attributes which do not lend themselves to casual determination of bicarbonate.

In this report, a rapid microdiffusional method is described for determining total CO_2 in small fluid volumes (10-100 ul) with a resolution of 6×10^{-7} equivalents. The relative advantages of this method over other published methods are its simplicity, the miniscule

investment in new apparatus, its high sensitivity and its suitability for adaptation as a laboratory exercise in an undergraduate physiology course. With only slight modification in procedure requiring minimal additional cost, the resolution of this method can be increased about 60 fold to 1×10^{-8} equivalents, while simultaneously remaining suitable for infrequent determination of 50 or fewer samples per day.

The method involves placing a sample in the center well of an Öbrink (1955) Modified Conway-Byrne (1933) microdiffusion dish and driving off the CO_2 by acidification with 0.1N HCl [Equation (1-1)].

$$HCO_3^- + H^+ \rightleftharpoons H_2CO_3 \longrightarrow H_2O + CO_2 \uparrow \qquad (1\text{-}1)$$

A barium hydroxide [$Ba(OH)_2$] solution present in the middle well of the chamber traps the liberated CO_2 as insoluble $BaCO_3$ [Equation (1-2)] with a consequent drop in pH of the unreacted $Ba(OH)_2$.

$$Ba(OH)_2 + CO_2\ (g) \longrightarrow BaCO_3 \downarrow + H_2O \qquad (1\text{-}2)$$

Since the number of equivalents of base required to restore the pH of the $Ba(OH)_2$ solution to its initial value is directly proportional to the number of equivalents of CO_2 trapped as $BaCO_3$, then one can readily determine the total CO_2 content of the sample by back titrating the absorbing solution of $Ba(OH)_2$ to its initial pH.

Through a knowledge of the original pH of the sample and by employing the Henderson-Hasselbalch equation [Equation (1-3)], the concentration of HCO_3^- in the sample can be determined.

$$pH = pK_a + \log \frac{[HCO_3^-]}{[H_2CO_3]} \qquad (1\text{-}3)$$

where pK_a is the pH at which the protonated and unprotonated species are present at equal concentrations. For carbonic acid that pH is 6.1.

II. MATERIALS AND METHODS

An Öbrink (1955) modified Conway and Byrne (1933) microdiffusion dish is employed. The function of each chamber of the Conway dish has been reversed in the microdiffusion procedure to be described. The middle chamber which is normally the site of reaction is now used as the absorbing site, while the converse is true regarding the center chamber. This reversal facilitates a 6 fold increase in the surface area of the $Ba(OH)_2$ solution (1.13 cm^2 vs 6.28 cm^2), thereby increasing the quantity of CO_2 that can be captured on the surface of the unstirred $Ba(OH)_2$ layer.

The three chambers of the Conway dish are filled as follows: center well, 10-100 microliters of sample or standard; middle well, a solution of $Ba(OH)_2$ at pH 11.00; and outer well, 0.5 ml of 0.10N HCl to seal the chamber. After the sample is added, the lid on the chamber is partly put in place and 0.5 ml of 0.10N HCl added to the sample or standard solution present in the center well, whose final pH is less than 1.5. The lid is quickly placed over the dish and rotated in the 0.10N HCl in the outer well to insure a proper seal. [All solutions must be prepared daily in freshly boiled glass distilled water.] The sample is then incubated for 50 minutes (see below) after which little

additional absorption by the $Ba(OH)_2$ appears to occur. The lid is then carefully removed and the content of the center well discarded to prevent contamination of the middle well contents during the back titration. [The contribution of CO_2 from the atmosphere or originating with the investigator to the number of equivalents needed to back titrate the sample will be discussed in the results section.]

The microdiffusion dish is then tilted ca. 30° so that the tip of a microburet (0.2 ml capacity with divisions of 0.2 microliters; Roger Gilmont Industries, Great Neck, NY) and a micro-pH electrode (1.2 mm bulb diameter, Model MI-410, Microelectrodes Inc., Londonderry, New Hampshire) can be immersed into the $Ba(OH)_2/BaCO_3$ solution in the middle well (Figure 1.1). The $Ba(OH)_2$ is then titrated with 0.1N NaOH with continuous manual stirring from its acidic or just slightly basic pH (range 6.2-8.4) back to pH's 10.2, 10.3 and 10.4. The pH change during the back titration is continuously monitored via the micro-pH electrode (connected to a pH meter equipped with an expanded scale), while the volume of base needed to elevate the pH to each of the three end points (i.e., pH 10.2 . . .) is recorded. The equivalents of CO_2 in each sample is then calculated from a standard curve (0.5-10.0 microequivalents of $NaHCO_3$).

III. RESULTS AND DISCUSSION

In the procedure outlined above, precautions were not taken to exclude atmospheric CO_2 or that originating with the investigator, since such precautions are unnecessary and would preclude the routine use of

Figure 1.1. Schematic representation of back titration following carbon dioxide release and absorption. The microdiffusion dish is uncovered following incubation and the lid set aside. The contents of the center well are removed to prevent contamination of that in the middle well and the dish tilted ca. 30° to force the absorbing solution of $Ba(OH)_2$ to collect at one side. While continuously monitoring the pH and manually swirling the absorbing solution within the dish, the contents are then back titrated with 0.1 N NaOH. [Inset. The tips of a micro-pH electrode and a micro-buret are placed under the surface of the absorbing solution.]

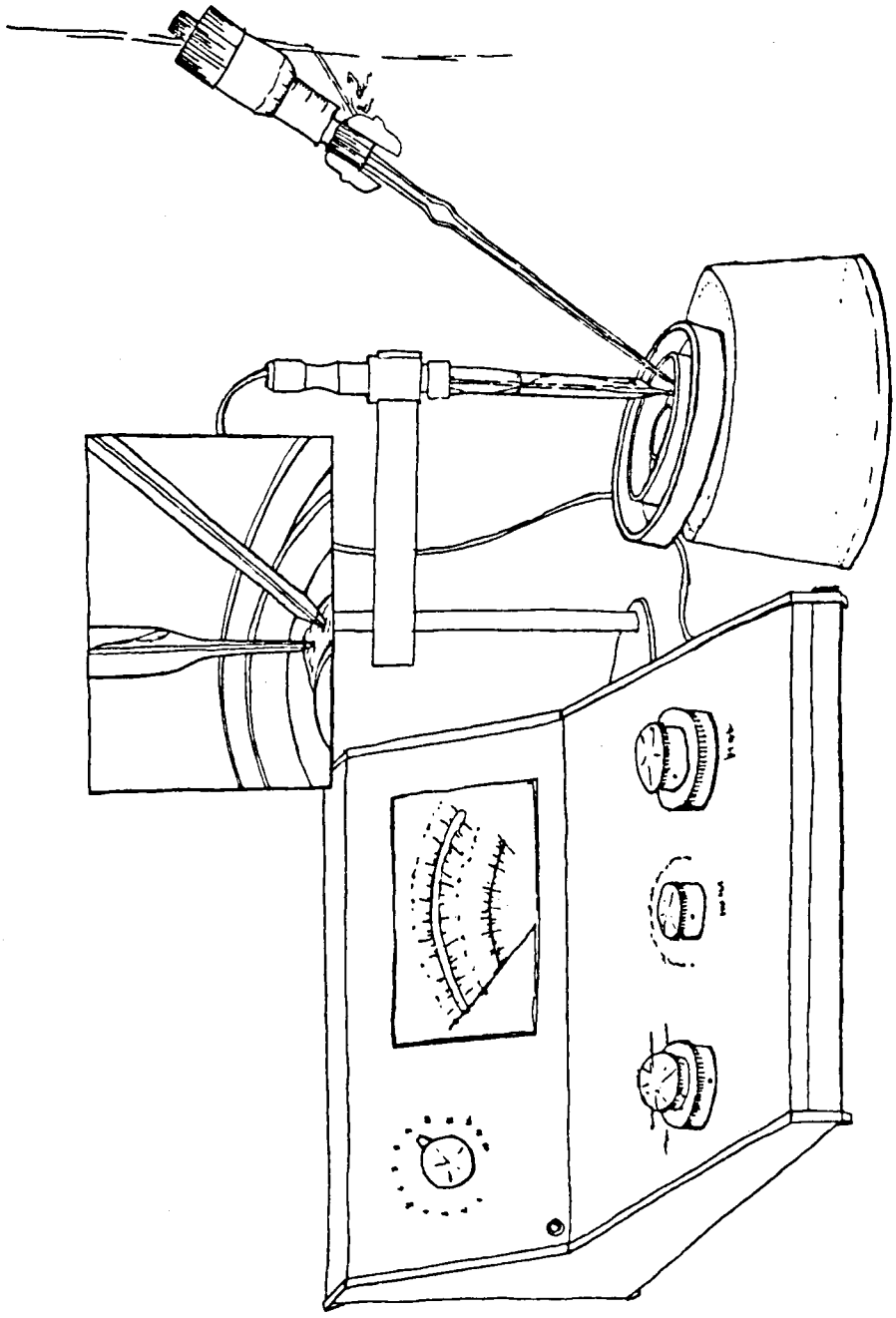


Figure 1.1

this procedure as a laboratory exercise in a student laboratory. Consequently, corrections were made for the effect of this incident CO_2 on the pH of the absorbing solution. One measure of CO_2 contamination during sample incubation is the change in pH of a "blank" dish containing only a solution of $Ba(OH)_2$. The concentration of CO_2 in the atmosphere is extremely variable, and the final pH of the $Ba(OH)_2$ solution in the presence or absence (= blank) of sample or standard solution will vary. Thus, following incubation of the blank, a range of values is obtained (pH 10.45-10.60) rather than a single ph value (i.e., a value indicative of the pH drop due to absorption of CO_2 from the air). This variability of blank pH's can create large errors during back titration, since enormous quantities of NaOH equivalents are needed to effect only small changes in pH of the $Ba(OH)_2$ solution at high pH end points (Figure 1.2). This problem can be circumvented by back titrating to pH values that are lower than those of the blank. This approach will preclude over-titrating the sample, thereby obviating the need to titrate to the "true" end point. Although this technique does reduce the incident recovery of CO_2, it in no way diminishes the overall advantage of the method.

The numbers of equivalents of base required following incubation to reach each of the three pH end points (10.2, 10.3 and 10.4) are read off of a standard curve and averaged to calculate the total number of equivalents present in the sample. Since one knows the efficiency of recovery at each HCO_3^- concentration (Figures 1.3 and 1.4) for each pH end point, the total number of equivalents in each sample can readily be calculated with great accuracy.

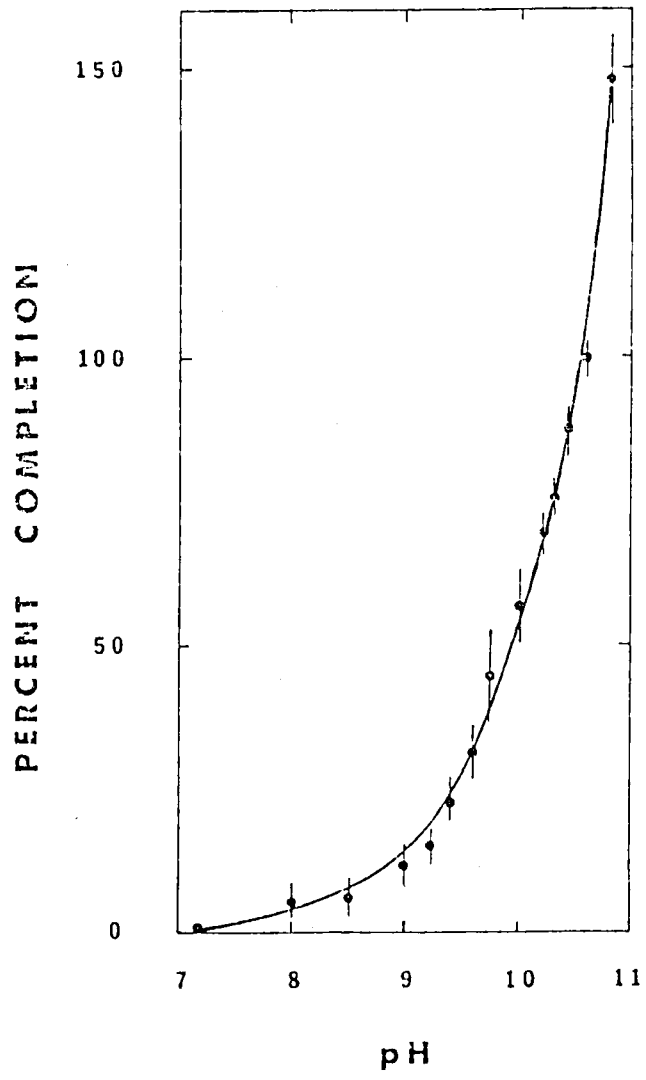


Figure 1.2. The relationship between the percentage of total bicarbonate recovered and the pH of the titrated solution. In four separate determinations, 10 uEq aliquots of bicarbonate were placed in the center well of a microdiffusion dish, the dish covered, and 0.1 ml of 0.5 N HCl added (final pH 1.5). Following fifty minutes of incubation, the absorbing solution 1.2 ml of $Ba(OH)_2$ plus sample or blank, initial pH 11.0, final pH 7.33 was back titrated with continuous monitoring of pH. The maximal recovery of 2.7 uEq (100%) was attained at pH 10.6, the pH of the blank (see Figure 1.7). Back titration above pH 10.6 introduces rather substantial errors with minimal changes in pH. Back titration of samples was normally terminated at pH 10.4 for this reason, even though the absolute efficiency of recovery, at pH 10.4, is less than that at pH 10.6.

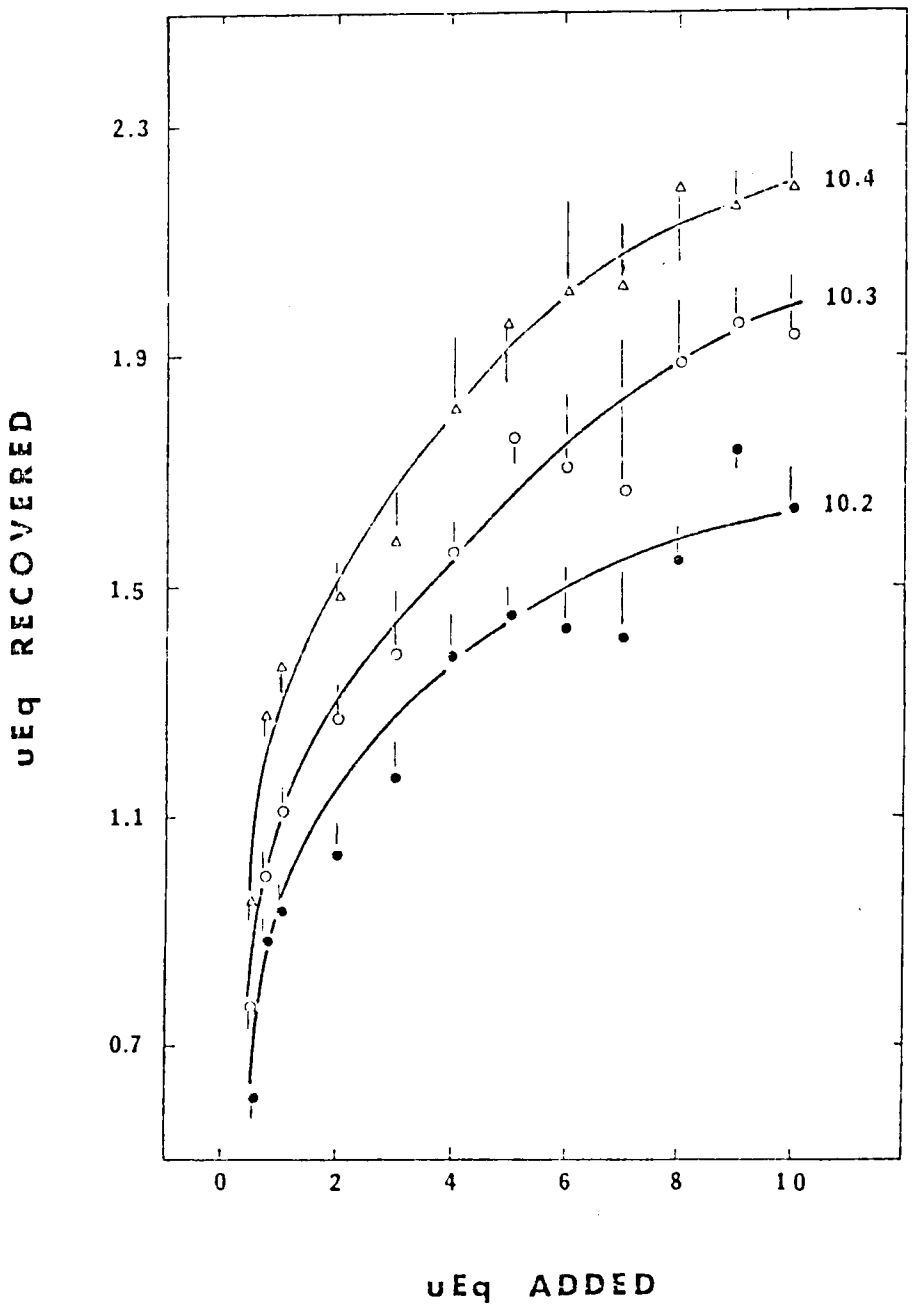


Figure 1.3. Recovery of bicarbonate at various pH end points following addition of variable quantities of bicarbonate. The numbers of equivalents of base needed to return the pH of the respective absorbing solutions (which had fallen during the incubation period to between 6.3-8.4) to pH's 10.2, 10.3 and 10.4 were determined. Each point is the mean of three experiments. Bars represent standard errors. (see legend in Figure 1.2 for details.)

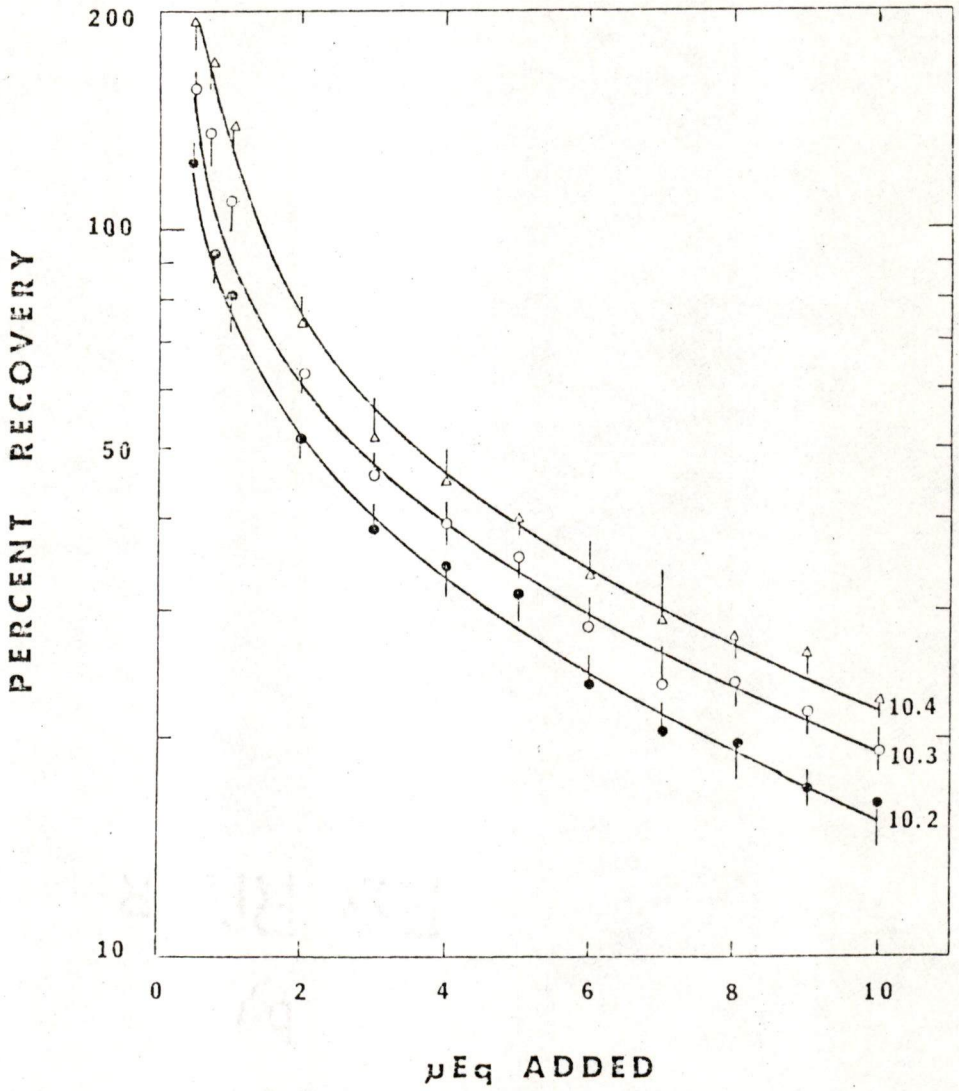


Figure 1.4. Percentage of added bicarbonate recovered at pH's 10.2, 10.3 and 10.4. Microequivalents of bicarbonate recovered at each pH (Figure 1.3) are plotted as percentages of the total quantities added. Recovery in excess of 100% represents contamination from ambient CO_2. Each point is the mean of three experiments.

The problem of pH changes in the absorbing $Ba(OH)_2$ solution originating from atmospheric CO_2 can be reduced by optimizing the number of equivalents of based employed in the absorbing solution (Figures 1.5-1.6). Large shifts in pH occur in the absence of sample when the number of $Ba(OH)_2$ equivalents is small [e.g., 1.2×10^{-6} when 0.3 ml of 2 mM $Ba(OH)_2$ is employed] (initial pH = 11.0, final pH = 6.0), while the presence of 8×10^{-6} or more equivalents of $Ba(OH)_2$ prevents detection of small quantities of CO_2 derived from the sample. Consequently, an intermediate number of equivalents (4.8×10^{-6} from 1.2 ml) was chosen to give a reasonable balance between sensitivity towards sample (0.6×10^{-6} equivalents; see Figure 1.3) and contaminants (initial pH 11.0, final pH = 10.6-10.45). Were precautions taken to carry out the entire procedure in a CO_2 free environment, the sensitivity towards bicarbonate in the sample would be increased by a factor of ca. 10. This ten fold increase is derived from a four fold decrease in the number of equivalents needed to effect a pH change (1.2 vs 4.8×10^{-6} equivalents) and an anticipated 2.5 fold increase in sensitivity by virtue of the greater pH change per CO_2 equivalents. The sensitivity of this procedure can be further increased if a carbon dioxide free environment is employed. Firstly, the pH of the $Ba(OH)_2$ could then be titrated back to its initial value instead of pH's 10.2-10.4, yielding an ca. 2 fold increase in sensitivity. Secondly, the concentration of $Ba(OH_2)$ used as the absorbing solution could be decreased from 2.0 mM to 0.2 mM (Figure 1.6) to yield a 3 fold increase in sensitivity. The overall increase in sensitivity arising from use of a CO_2 free environment would

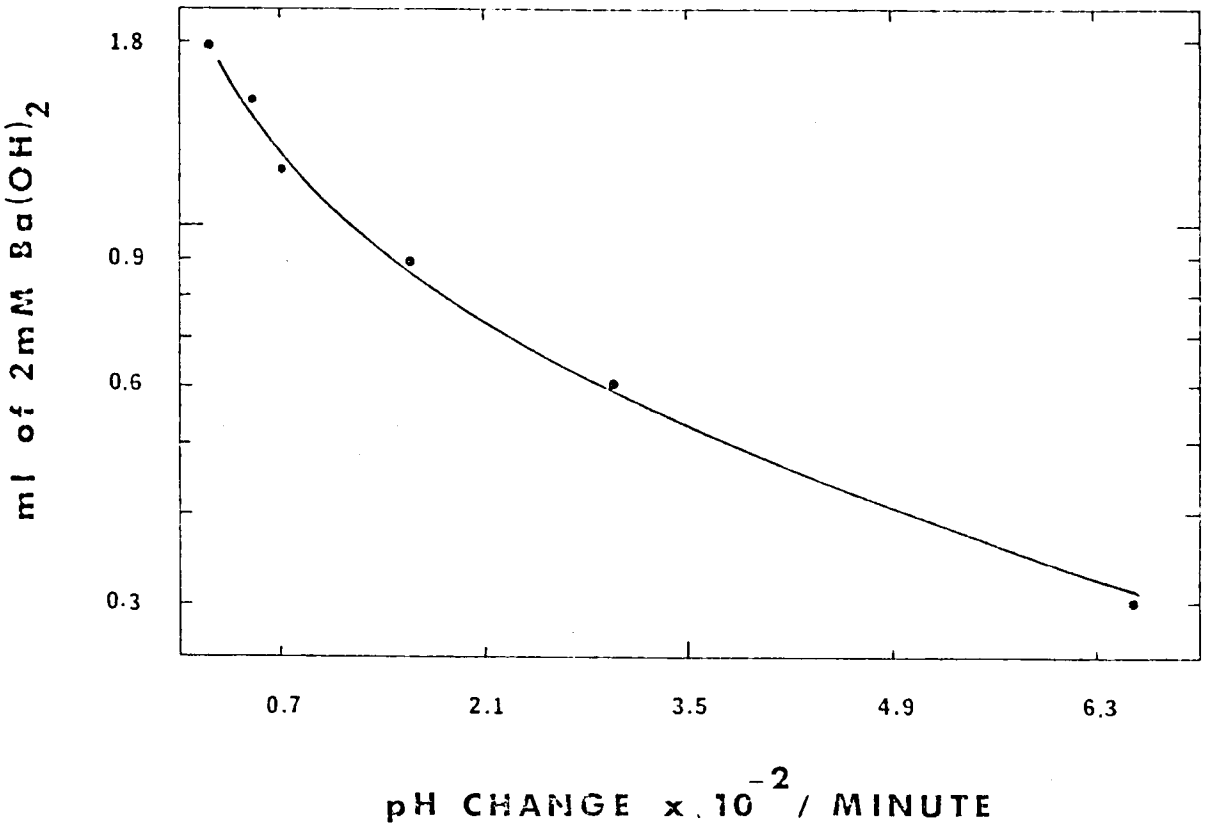


Figure 1.5. The effect of absorbing fluid volume on the pH change of a blank solution of 2.0 mM $Ba(OH)_2$. The decline in incident pH (beginning at pH 11.0) over a 100 minute period results from CO_2 absorption from the air. An intermediate volume of absorbing solution (1.2 ml of 2.0 mM) was chosen to reduce the effects of incident CO_2 contamination (less than 0.5 pH unit change/50 minutes), while simultaneously optimizing the systems sensitivity. Standard errors from triplicate samples were less than 1.5% of the means.

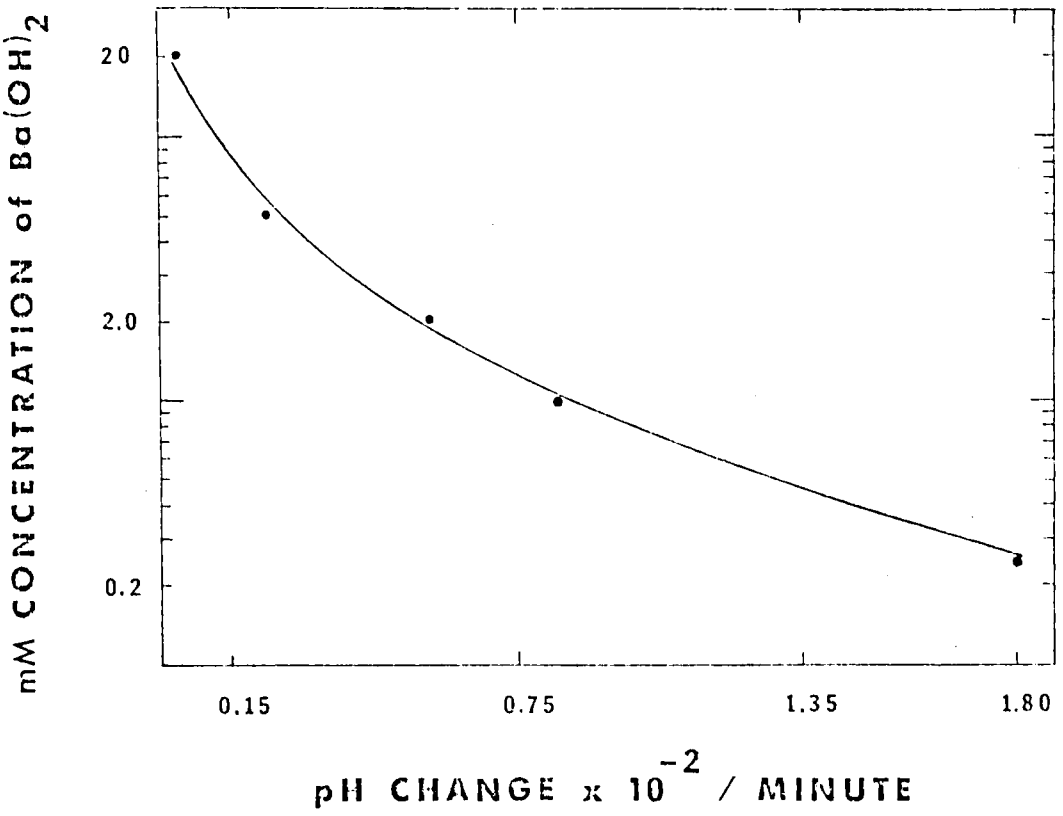


Figure 1.6. The effect of absorbing fluid concentration on the pH change of a blank. Triplicate aliquots of 1.2 ml of 0.2 mM to 20 mM concentrations of $Ba(OH)_2$ (initial pH 11.0) were incubated in micro-diffusion dishes for 25, 50 or 75 minutes and changes in pH determined. The change in pH at each concentration was linear throughout the 75 minutes of incubation. Each point is the mean of 9 determinations. Standard errors are less than 1.5% of the means.

be 10 × 2 × 3 = ca. 60 fold. Thus, the sensitivity of this method could be increased from a minimum of 6×10^{-7} equivalent to 1×10^{-8} equivalents.

When the method is carried out without taking special precautions regarding CO_2 in air, the efficiency of CO_2 capture from the sample remains constant following 50 minutes of incubation. When the numbers of microequivalents of HCO_3^- are 2.5 or less, the percentage recovery reaches a maximum after only 30 minutes of incubation, whereas the percentage recovery of larger samples (e.g., 10 microequivalents) is not optimized before 50 minutes (Figure 1.7).

The absolute numbers of microequivalents recovered for each standard were determined after constancy of recovery was attained (Figure 1.3). The percentage efficiency of recovery at each concentration of standard was then calculated (Figure 1.4). The variability in percentage efficiency of recovery for each quantity of HCO_3^- added is a measure of the extent to which a $Ba(OH)_2$ solution will absorb CO_2 released from the sample (Equation 1-2), as well as a function of the CO_2 gas tension in the micro-diffusion chamber. Since the $Ba(OH)_2$ solution is unstirred, a $BaCO_3$ saturated layer develops at the interface between the $Ba(OH)_2$ and the CO_2 gas, which inhibits further absorption. This unstirred layer places an effective upper limit on the absolute amount of CO_2 which can be recovered per unit surface area of $Ba(OH)_2$. That 100% efficiency of recovery is not reached at concentrations of standard below this upper limit suggests that the CO_2 tension in the diffusion dish is not high enough to drive CO_2 across the $Ba(OH)_2$

Figure 1.7. The efficiency of recovery of 2.5 uEq and 10.0 uEq of bicarbonate following variable periods of incubation. The evolved CO_2 was trapped in 1.2 ml of 2.0 mM $Ba(OH)_2$ [initial pH 11.0], and the quantity captured determined every five minutes during the first 20 minutes of incubation, and every ten minutes between 20-100 minutes of incubation. Absorbing solutions were back titrated to pH 10.6, that of the blank, rather than 11.0, that of the initial pH. Each point is the mean of duplicate analyses. Standard errors (N = 24) of 3.5 ± 0.7% (equivalent to 0.07 uEq) and 8.0 ± 1.4% (equivalent to 0.22 uEq), respectively, were determined for the 2.5 and 10 uEq standards.

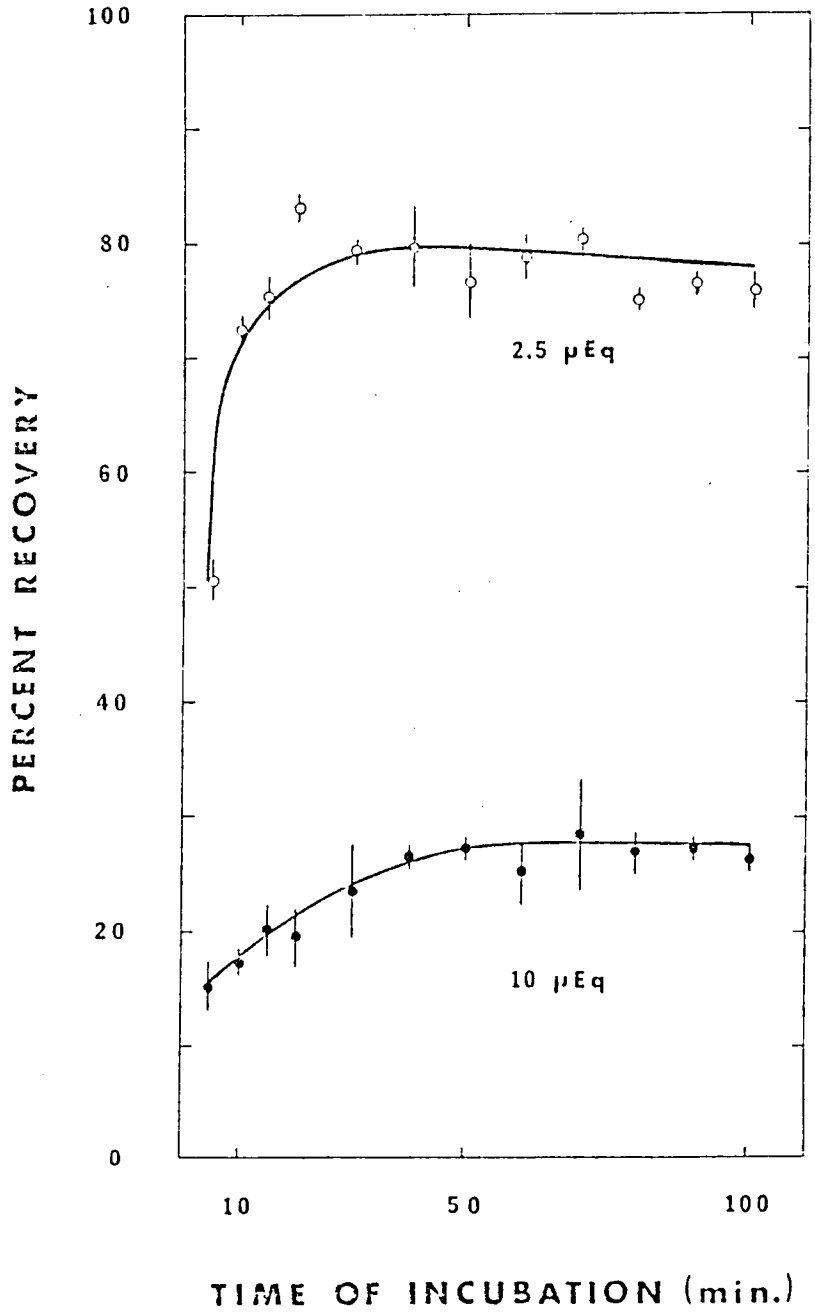

100
80
60
40
20
0
PERCENT RECOVERY
2.5 µEq
10 µEq
10
50
100
TIME OF INCUBATION (min.)

Figure 1.7

interface. Levels of recovery above 100% efficiency can be attributed to back titrating with the samples the incidental CO_2 present in the air when the chamber was sealed. Although limitations of CO_2 diffusion prevent 100% efficiency at all concentrations of bicarbonate, the precision of the method is unaffected. Further, the exact percentage recovered (i.e., accuracy) is known for each concentration of standard.

IV. SAMPLE CALCULATION

An example using this method to determine the concentration of bicarbonate in a biological sample is as follows:

1. 100 ul of molting fluid (pH 7.23) is placed in the dish's center well and acidified with 0.5 ml of 0.10 N HCl.
2. 1.2 ml of 2 mM $Ba(OH)_2$ is placed in the middle well to absorb the released CO_2. The initial pH of the trapping solution is 11.04. The pH following 50 minutes of incubation in the sealed microdiffusion dish is 7.56.
3. The absorbing solution is then back titrated with 0.10 N NaOH.

Volume of Base Required	Normality of Base × Volume of Base = uEq of CO_2 Recovered	uEq Added (Fig. 1.3, p. 13)
15.8 ul to pH 10.2	1.58	7.9
18.9 ul to pH 10.3	1.89	8.0
21.5 ul to pH 10.4	2.15	8.2
		$\bar{x}$ = 8.03

8.03 uEg/0.1 ml = 80.3 mM total CO_2 captured.

Physically dissolved CO_2 = 0.76 mM at 25°C (Handbook of Chemistry and Physics, 1964, 45th Ed., Chemical Rubber Co., Cleveland), leaving

79.6 mM CO_2 as carbonic acid + bicarbonate. The relative contribution of each is determined from the Henderson-Hasselbalch equation [Equation (1-3)]. Substituting known values, one gets

$$7.23 = 6.1 + \log \frac{[HCO_3^-]}{[79.6 - HCO_3^-]} \quad (1\text{-}4)$$

$$1.13 = \log \frac{[HCO_3^-]}{[79.6 - HCO_3^-]} \quad (1\text{-}5)$$

$$13.48 = \frac{[HCO_3^-]}{[79.6 - HCO_3^-]} \quad (1\text{-}6)$$

Therefore, the concentration of HCO_3^- in moulting fluid is 74.1 mM and the concentration of carbonic acid is 5.5 mM.

V. SUMMARY STATEMENT

A microdiffusion method is described which permits the determination of 6×10^{-7} equivalents of bicarbonate in biological fluids. CO_2 in the sample is driven off with 0.1 N HCl and captured by 1.2 ml of 2 mM $Ba(CO)_2$ (initial pH 11.0). Since incident CO_2 is also captured by the $Ba(OH)_2$, the absorbing solution is back titrated to fixed points (10.2-10.4) lower than the initial pH or 11.0, yielding up to 80% sample recovery. The concentration of total CO_2 in the samples can then be determined from a knowledge of the quantity of CO_2 captured when samples of known concentrations are used. The proportion of the total CO_2 present as HCO_3^- is determined from a knowledge of the sample pH and by application of the Henderson-Hasselbalch equation.

CHAPTER 2

CHANGES IN BICARBONATE, CHLORIDE, AND HYDROGEN ION DISTRIBUTIONS IN HEMOLYMPH AND MOULTING FLUID DURING THE LARVAL-PUPAL TRANSFORMATION OF THE TOBACCO HORNWORM (Manduca sexta)

I. INTRODUCTION

Although the composition of insect hemolymph has been extensively studied (see reviews by Chauvin, 1956; Duchateau, et al., 1953; and Florkin and Jeuniaux, 1974), very little attention has been paid to hemolymph bicarbonate and pH (see, however, Buck, 1953; Jungreis, 1978; and Wyatt, 1961). Bicarbonate (and for that matter H^+ ion) has been implicated in various physiological systems in insects (moulting fluid formation, Jungreis, 1979, and Jungreis and Harvey, 1975; labial gland secretion, Kafatos, 1975; and rectal resorption of water, Phillips, 1977, and Phillips, et al., 1977), yet no extensive study of the changes in hemolymph HCO_3^- and pH over an extended portion of insect development has been undertaken. Indeed only a few studies on hemolymph bicarbonate at only one stage in development have been undertaken (see Buck and Friedman, 1950; Levenbrook, 1950; Buck, 1958; and Levy and Schneiderman, 1958) and none on the HCO_3^- concentration in moulting fluid.

Heimple (1955, 1956), working with the larch sawfly Pristiphora erishonia (Hartwig); Demjanowski, et al. (1932), working with Bombyx morii; and Bishop (1953), working with the honeybee, have reported

transient pH rises in hemolymph during larval-pupal moulting. Very few workers, however, have investigated the changes in pH in both hemolymph and moulting fluid during the larval-pupal transformation (Jungreis, 1978; Passonneau and Williams, 1953; and Katzenellenbogen and Kafatos, 1970).

In this paper I report the changes in HCO_3^-, Cl^-, and pH in hemolymph and moulting fluid during the larval-pupal transformation in the tobacco hornworm, Manduca sexta. In addition to quantifying moulting fluid HCO_3^- for the first time, I also offer the probable sites of its formation (see also Johnston and Jungreis, 1979). Furthermore, I show that these changes in ionic composition are intimately associated with the moulting process.

II. MATERIALS AND METHODS

Experimental Animals

Tobacco hornworms used in these experiments were obtained from an inbred colony maintained in our laboratory. Larvae were reared on the wheat germ-casein diet of Bell and Joachim (1976) under an 18L:6D photoperiod regimen at 23 to 25°C. Animals were staged and hemolymph and moulting fluid collected according to the method of Jungreis (1978).

Analysis of Bicarbonate and Chloride

The concentration of chloride in hemolymph and moulting fluid was determined coulometrically with a Buchler-Cotlove Chloridometer (Model 4-2008; Buchler Instruments, Fort Lee, New Jersey). Duplicate 10 or

20 µl samples were analyzed with better than ± 5% agreement between duplicate analyses.

Analysis of bicarbonate was via the micro-diffusional method of Johnston and Jungreis (1978).

Analysis of pH

The pH of moulting fluid and hemolymph was determined with a miniature combination pH electrode (1.2 mm bulb diameter, Model MI-410, Microelectrodes Inc., Londonderry, New Hampshire) connected to a Corning Model 12 pH meter (read on expanded scale). The pH of hemolymph and moulting fluid was determined in situ and following collection via the method of Jungreis (1978).

III. RESULTS

Bicarbonate in Hemolymph

Bicarbonate levels in M. sexta hemolymph fall gradually during the first three days of the larval-pupal transformation from a high of 4.3 mM (Table 2.1, Figure 2.1). During the next 24 hour period (between pink stripe + 3 days and early moulting fluid) there is a marked increase in the concentration of Cl^- from 25 mM at pink stripe + 3 days to 31 mM at early moulting fluid (Table 2.1, Figure 2.1). Thereafter Cl^- drops to around 22 to 24 mM in middle and late moulting fluid stages, respectively.

Chloride in Moulting Fluid

The chloride concentration in moulting fluid is maintained between 5 and 7 mM (Table 2.1, Figure 2.1) during the entire moulting fluid

TABLE 2.1. The concentration of bicarbonate, chloride, and hydrogen ion in hemolymph and moulting fluid in the tobacco hornworm, Manduca sexta.

Stage of Development	Hemolymph			Moulting Fluid		
	mM HCO_3^-	mM Cl^-	pH	mM HCO_3^-	mM Cl^-	pH
Feeding Fifth	4.32±0.25 n=7	23.5±3.1 n=7	6.75±.03 n=7			
Pink Stripe	3.22±0.63 n=4	22.5±4.6 n=3	6.65±.02 n=6			
Pink Stripe + 2 Days	1.79±0.15 n=3	25.0±2.3 n=3	6.72±.02 n=5			
Pink Stripe + 3 Days	5.5±3.16 n=4	25.4±2.4 n=3	6.62±.01 n=5			
Early Moulting Fluid	8.85±3.44 n=3	31.3±2.3 n=3	6.69±.03 n=8	71.3±27.8 n=5	7.37±0.62 n=3	7.28±.030 n=4
Middle Moulting Fluid	11.15±5.9 n=7	22.3±1.2 n=3	6.68±.03 n=10	84.2±19.6 n=7	5.30±1.87 n=4	7.39±.034 n=8
Late Moulting Fluid	9.78±3.74 n=5	24.7±1.8 n=5	6.66±.02 n=5	99.5±31.3 n=5	6.15±1.11 n=5	7.36±.072

± = the standard error of the mean.

n = the number of individual observations.

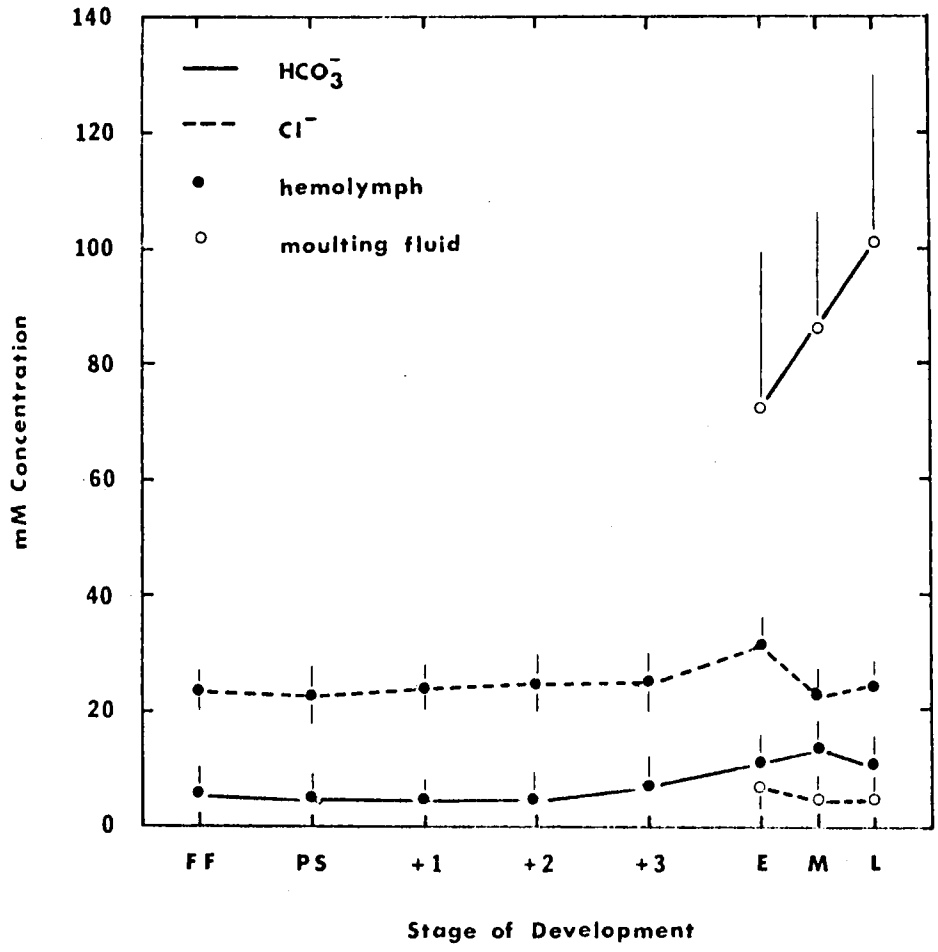


Figure 2.1. Bicarbonate and chloride levels in hemolymph and moulting fluid in Manduca sexta during the larval-pupal transformation.

process. The moulting fluid/hemolymph chloride ratio, unlike that of bicarbonate, is on the order of .23 to .25 (compared to 8.7 to 12.6 for HCO_3^-, see Table 2.2).

pH of Hemolymph

During the larval-pupal transformation the hemolymph is a slightly acidic (pH ca. 6.6) fluid. This pH is maintained within very narrow limits (6.75 during the fifth instar to 6.62 at pink stripe + 3 days, Table 2.1). During the period of moulting fluid formation the hemolymph pH remains slightly acidic and within the same narrow range (6.66 at early moulting fluid, 6.68 during middle moulting fluid and 6.68 during late moulting fluid, Table 2.1). In fact, during the period of moulting fluid secretion and resorption the pH of hemolymph is practically a constant 6.66.

pH of Moulting Fluid

Moulting fluid, formed during the latermost stages of the larval-pupal transformation, is, unlike hemolymph, a slightly basic (pH ca. 7.3) potassium bicarbonate salt solution (see Jungreis and Harvey, 1975; and Jungreis, 1978). During the secretory phase of the moulting fluid process the pH rises from 7.28 (early) to 7.39 (middle, Table 2.1). During the late (i.e., resorptive phase) the pH of moulting fluid is maintained around 7.36 (Table 2.1).

TABLE 2.2. Ratios of bicarbonate and chloride in moulting fluid and hemolymph during early, middle and late moulting fluid stages. Numbers are averages of paired data ± the standard error.

	Moulting Fluid Stages		
	Early	Middle	Late
		Bicarbonate	
Moulting Fluid / Hemolymph	8.7±2.7 n=4	10.2±3.1 n=6	12.6±2.5 n=5
		Chloride	
Moulting Fluid / Hemolymph	.235±.026 n=3	.238±.048 n=2	.249±.017 n=5

n = number of individual determinations.

IV. DISCUSSION

Relationship between Hemolymph HCO_3^- and Cl^- Prior to Moulting Fluid Formation

The HCO_3^- present in hemolymph originates from the action of the enzyme carbonic anhydrase, present in either the fat body of integument (other obvious sources being devoid of the enzyme, see Johnston and Jungreis, 1979). Hemolymph Cl^- is derived from the diet, the concentration of Cl^- in the food is approximately 35 mM (Jungreis, Jatlow and Wyatt, 1973).

The concentration of HCO_3^- in hemolymph decreases during the first three days (4.3 mM at feeding fifth to 1.8 mM at pink stripe + 2 days) of the larval-pupal transformation. I have found (Johnston and Jungreis, 1979) that during this period the activity of carbonic anhydrase in both fat body and integument decreases by ca. 50%, thus accounting for the reduced HCO_3^-. Hemolymph HCO_3^- levels begin to increase between pink stripe + 2 and pink stripe + 3 days (see Table 2.1), while no corresponding increase in carbonic anhydrase activity was seen between these stages. Indeed, I have found that fat body carbonic anhydrase activity remains relatively constant from pink stripe + 2 days until pupation. However, between pink stripe + 2 days and pink stripe + 3 days there is a significant increase in the number of HCO_3^- equivalents (Table 2.3). This increase in HCO_3^- equivalents accounts for the entire increase in the hemolymph concentration of HCO_3^- (hemolymph volume decreases only slightly between these stages, see Boyce, 1977). The question remains, however, as to the source of the increase in the

TABLE 2.3. The concentration of chloride, bicarbonate, and hydrogen ion in hemolymph and moulting fluid of lab reared silkmoths, Manduca sexta.

	FF	PS	PS+2	PS+3	E	M	L
				Bicarbonate Equivalents $\times 10^{-6}$			
Hemolymph	16	13	5	14	18	20	16
Moulting Fluid	--	--	-	--	4	21	5
Total	16	13	5	14	22	41	21
				Chloride Equivalents $\times 10^{-6}$			
Hemolymph	86	89	69	66	63	40	40
Moulting Fluid	--	--	--	--	.4	1	.3
Total	86	89	69	66	63.4	41	40.3

FF = feeding fifth.

PS = pink stripe.

PS+2 = pink stripe + 2 days.

PS+3 = pink stripe + 3 days.

E = early moulting fluid.

M = Middle moulting fluid.

L = Late moulting fluid.

number of HCO_3^- ions between pink stripe + 2 and pink stripe + 3 days. Carbonic anhydrase present in the fat body does not undergo any increase in activity between these stages; in fact, total carbonic anhydrase/ animal actually declines until pink stripe + 3 days. The source of the increased HCO_3^- in hemolymph between these stages is as yet unclear. Changes in overall metabolism coupled with the disruption of the tracheal continuity at apolysis could account for an increase in CO_2 with a corresponding increase in hemolymph bicarbonate.

During the first 4 days of the larval-pupal transformation (feeding fifth to pink stripe + 3 days) hemolymph Cl^- levels are maintained rather stably around 25 mM (see Figure 2.1, p. 27). At this time (pink stripe + 3 days) there is a 20% increase in the hemolymph Cl^- concentration (25 mM at pink stripe + 3 days to 31 mM at early moulting fluid, see Figure 2.1). This increase in due entirely to a decrease in the volume of hemolymph. At the time of the transformation between pink stripe + 3 days and early moulting fluid there is a 23% decrease in the hemolymph volume (2.60 ml at pink stripe + 3 days to 2.01 at early moulting fluid, Boyce, 1978). This decrease in fluid volume is brought about by movement of water out of the hemolymph, across the integument and into the exuvial (or moulting space) as moulting fluid. Since moulting fluid has a relatively low chloride content with respect to hemolymph (see Table 2.1, p. 26), the increase in hemolymph Cl^- is due entirely to a decline in fluid volume. Because the absolute number of hemolymph chloride equivalents does not change between pink stripe + 3 days and early moulting fluid (66×10^{-6} Eqs. at pink stripe + 3 days

to 63×10^{-6} Eqs. at early moulting fluid, see Table 2.3) the increase in hemolymph Cl^- concentration attests to the effective exclusion of hemolymph Cl^- from moulting fluid.

Despite the fact that different physiological processes control the levels of HCO_3^- and Cl^- in hemolymph, the levels of the two ions appear to change proportionately (see Figure 2.2). Furthermore, Cl^- makes up the largest bulk (ca. 90%) of the Cl^- + HCO_3^- total, and this percentage is maintained rather constantly (i.e., parallels the line of equality, see Figure 2.3, Line A) throughout the entire period preceeding moulting fluid formation. Though the controlling processes are different, they are certainly not separate. In fact, we have found (Johnston and Jungreis, 1979) that fat body carbonic anhydrase is inhibited (40%) by chloride concentrations in the range of 10 to 50 mM.

Relationship between Hemolymph HCO_3^- and Cl^- During Moulting Fluid Stages

The increase in hemolymph HCO_3^-, which began between pink stripe + 2 days and pink stripe + 3 days, continues until the middle moulting fluid stage. Dating periods of moulting fluid formation hemolymph HCO_3^- levels range around 10 mM (early, middle and late moulting fluid stages are not significantly different from one another, $p > .05$, but they are significantly different from pink stripe + 2 days, $p < .01$; see Tables 2.1 and 2.3). Between late moulting fluid resorption and pupation the hemolymph HCO_3^- level declines to ca. 5 mM and remains at this level throughout the pupal-adult metamorphosis (data not shown).

During the period between early and middle moulting there is a dramatic increase in the activity of integumentary carbonic anhydrase

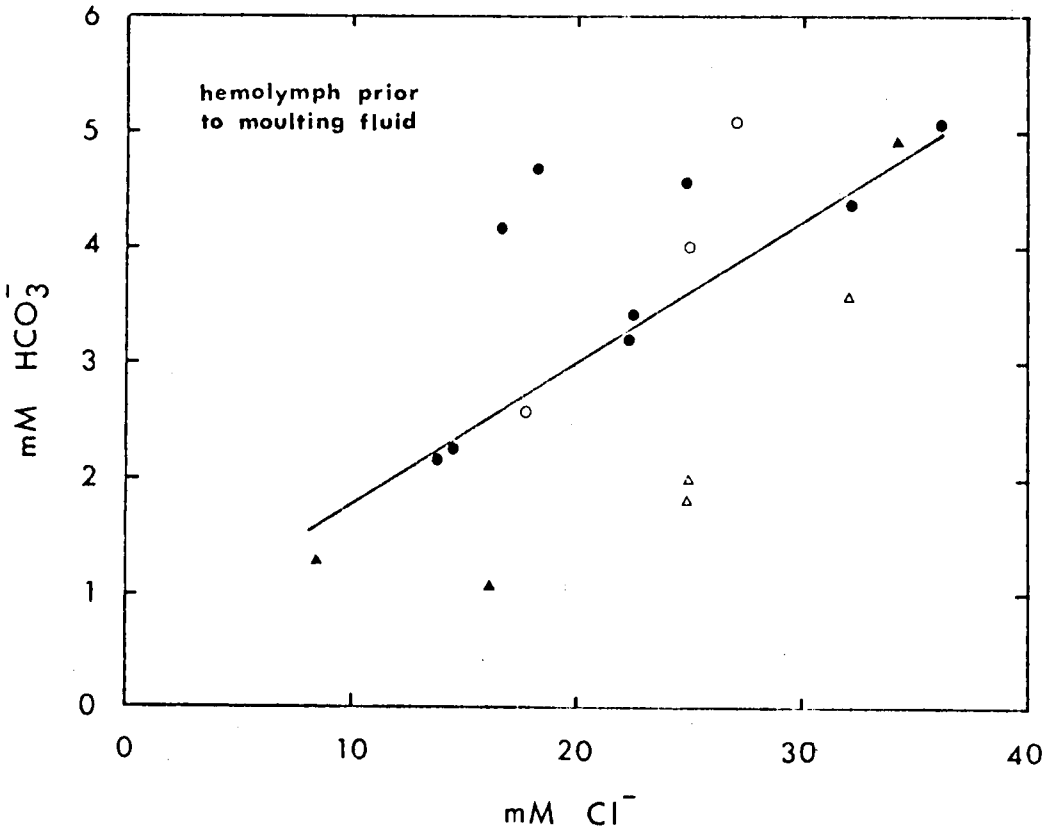


Figure 2.2. Relationship between bicarbonate and chloride in hemolymph prior to moulting fluid elaboration. ● = feeding fifth; O = pink stripe; ▲ = pink stripe + 2 days; Δ = pink stripe + 3 days.

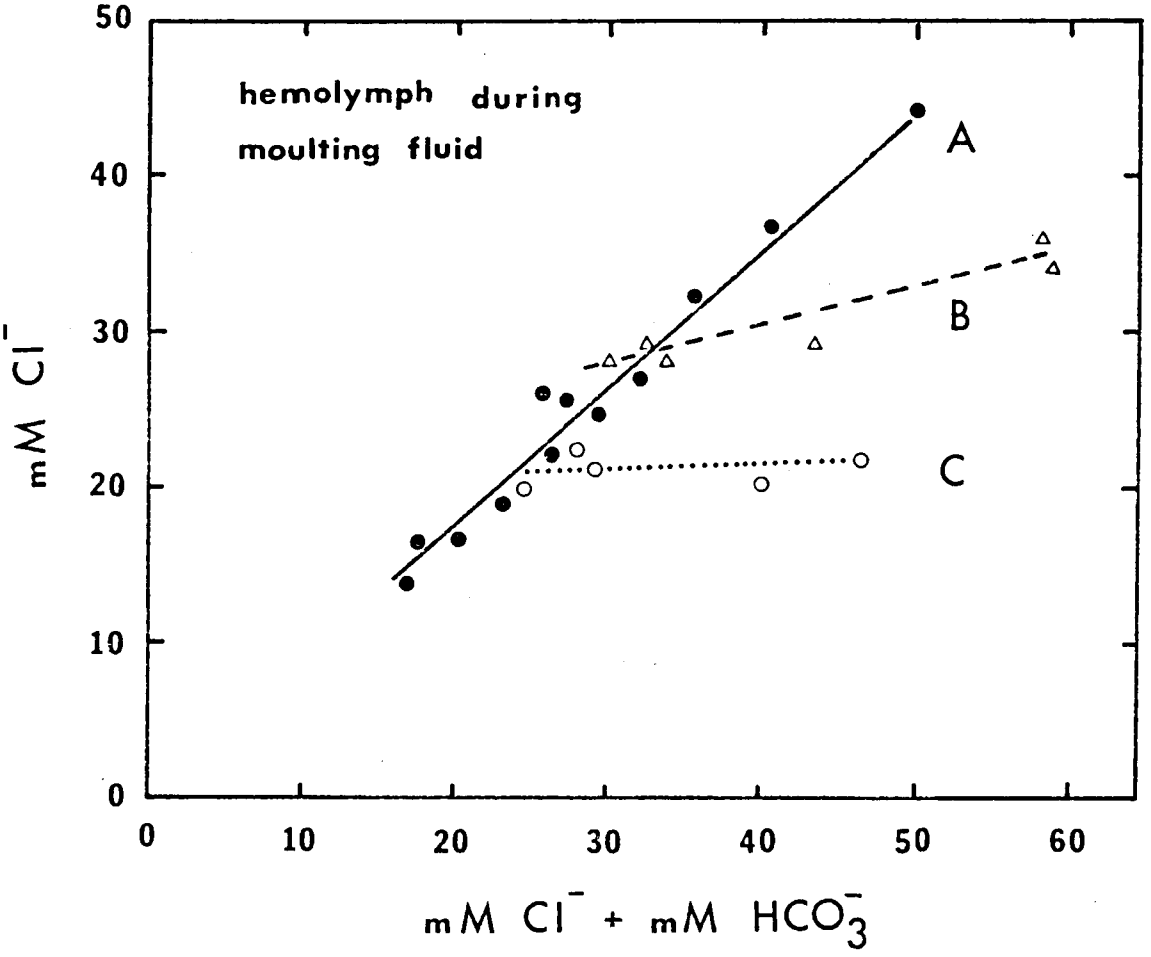


Figure 2.3. Relationship between hemolymph chloride and bicarbonate during moulting fluid secretion and resorption. A = relationship prior to moulting; B = relationship during early secretion and late resorption; and C = relationship during middle secretion and resorption.

(Johnston and Jungreis, 1979). It is believed that the primary result of this increased enzymatic activity is the production of bicarbonate destined for moulting fluid. The bicarbonate appearing in moulting fluid accompanies actively transported potassium (Jungreis, 1979) and acts to preserve electroneutrality. However, some of this newfound wealth of HCO_3^- may also find its way into hemolymph, thus helping to account for the increase in hemolymph HCO_3^- seen during moulting fluid formation.

As previously mentioned, hemolymph chloride levels are elevated during early moulting fluid with respect to the preceeding stages (see Table 2.1, p. 26). Between early and middle moulting fluid both Cl^- concentration and hemolymph volume begin to fall (Cl^- from 31 to 22 uM, and hemolymph volume by 10%). The decline in Cl^- is reflected in a decrease (33%) in the absolute number of Cl^- equivalents present between early moulting fluid and middle moulting fluid (see Table 2.3, p. 31). The number of Cl^- equivalents in hemolymph remains constant thereafter (Table 2.3), with the increase in concentration at late moulting fluid (Table 2.1; Figure 2.1, p. 27) reflecting the continued loss of hemolymph volume (1.81 ml at middle moulting fluid to 1.61 ml at late moulting fluid, see Boyce, 1977). The fate of the chloride ions lost from hemolymph between early and middle moulting fluid stages is not certain. A small portion (1 to 2%) of these ions are moving into moulting fluid, but with a total decrease in excess of 35%, this movement of Cl^- is practically insignificant. It is possible that a portion of the Cl^- lost from hemolymph could be moving into the gut or

other tissue via some storage process, but this is not known at this time.

The proportionate relationship between HCO_3^- and Cl^- in hemolymph (with Cl^- contributing ca. 90% of the total), present during the stages preceeding moulting fluid (see Figure 2.3, Line A), changes drastically as the animal reaches the moulting fluid stages. Cl^- no longer continues to account for a constant percentage of the total, because the higher rate of HCO_3^- production (relative to that of Cl^-) begins to assert itself (Figure 2.3, Line B). As the animal moves into middle moulting fluid the chloride levels decrease (with respect to early moulting fluid) while HCO_3^- values continue to increase. This relationship gives HCO_3^- its largest contribution to the total (HCO_3^- + Cl^-) and produces a correlation line between Cl^- and the HCO_3^- + Cl total with no slope (Figure 2.3, Line C).

As the animal nears the end of the period in which it is actively resorbing moulting fluid, the relationship between Cl^- and HCO_3^- merely retraces its steps backwards, but for different reasons. Since Cl^- remains relatively constant (see Table 2.1, p. 26) its increased proportion to the total HCO_3^- + Cl^- must be due to a decrease in HCO_3^-. This is the case (see Table 2.1; Figure 2.1, p. 27; and Line B of Figure 2.3). The decline in hemolymph HCO_3^- at late moulting fluid and post-resorptive stages is due to a decline in the activity of carbonic anhydrase in all tissues (Johnston and Jungreis, 1979). It is interesting to note that the relationships present during early and late moulting fluid have a common line (Figure 2.3, Line B), even though the

HCO_3^- trends at the two stages are opposite (HCO_3^- increases during early moulting fluid and falls during late).

Relationships between Moulting Fluid HCO_3^- and Cl^-

Bicarbonate levels in M. sexta moulting fluid are approximately 10 × as high as those found in hemolymph at comparable stages (Table 2.2, p. 29). At the onset of moulting fluid secretion the concentration of HCO_3^- in moulting fluid is ca. 70 mM. The HCO_3^- levels rise throughout the moulting fluid process to a high of nearly 100 mM at late moulting fluid (see Figure 2.4).

The HCO_3^- present in moulting fluid probably does not come from hemolymph sources. Examination of Table 2.3 (p. 31) reveals that the total number of bicarbonate equivalents present in hemolymph prior to moulting fluid formation is only 18 μEqs. At the middle moulting fluid stage there are 21 uEqs. of HCO_3^- present in moulting fluid. In order to account for this level in moulting fluid the entire quantity of hemolymph HCO_3^- would have to be equilibrated across the integument without an accompaning increase in fat body carbonic anhydrase activity (Johnston and Jungreis, 1979). I have found that the integument itself is a potent source of the enzyme carbonic anhydrase (ca. 75% of the animal's total) and propose that the integument (and not fat body or other obvious sources) accounts for the bicarbonate in moulting fluid.

There are, therefore, two separate bicarbonate pools—one associated with hemolymph (derived from fat body carbonic anhydrase) and one associated with moulting fluid (derived from integumentary carbonic anhydrase).

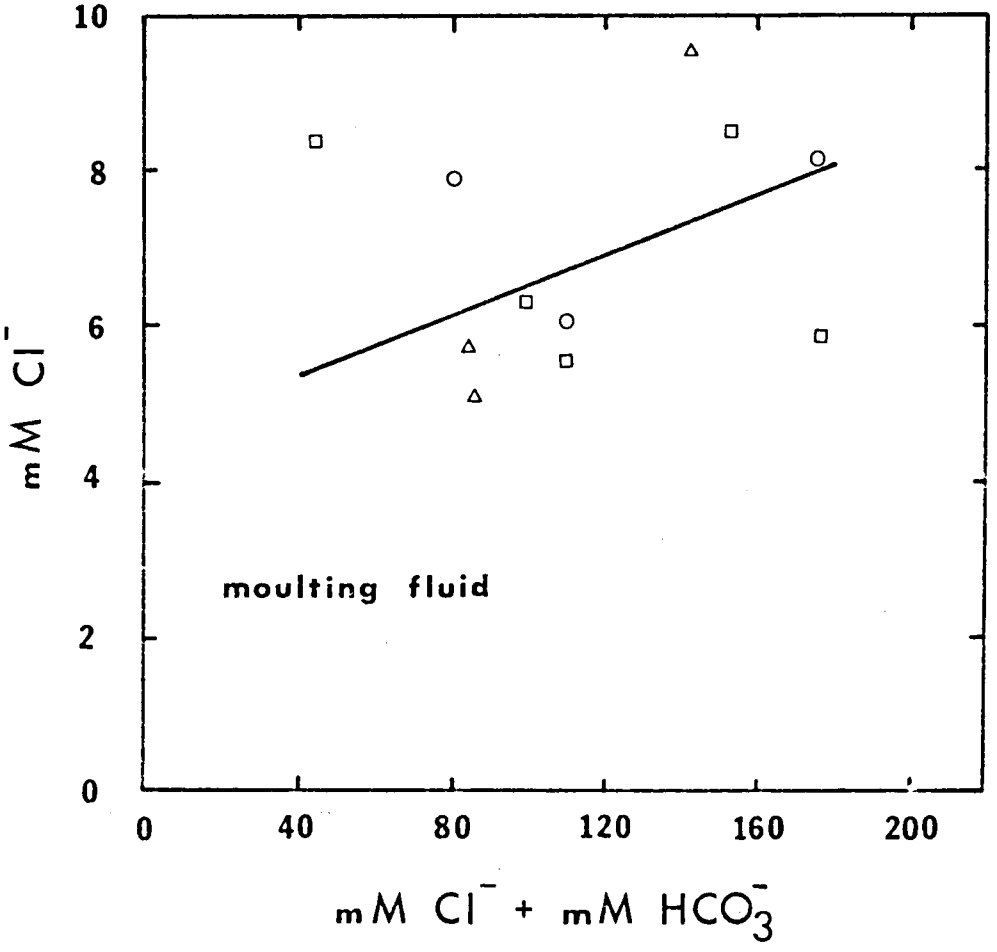


Figure 2.4. Relationship between chloride and bicarbonate in moulting fluid.

Chloride levels in moulting fluid, unlike those in hemolymph, are extremely low (less than 7 mM throughout the entire moulting fluid process, see Figure 2.1, p. 27, and Table 2.1, p. 26). The paried moulting fluid/bicarbonate ratio Cl^- is opposite that of HCO_3^- (ca. .23 for Cl^- vs. ca. 10 for HCO_3^-, see Table 2.2, p. 29). The concentration gradient of Cl^- between hemolymph and moulting fluid attests to the fact that the integumentary epithelium is an effective barrier to diffusion. In fact, Jungreis (1978) has found that in the absence of an osmotic differential (hemolymph and moulting are iso-osmotic, ca. 320 mOsms) and with significant differences in the concentrations of trehalose, glucose, phosphates, polypeptides and amino acids between moulting fluid and hemolymph there is no appreciable diffusion across the pharate pupal integument. The differences in Cl^-, HCO_3^- and pH between hemolymph and moulting fluid, reported here, further substantiate the notion that the integument is an effective barrier to diffusion.

CHAPTER 3

CHANGES IN BICARBONATE, CHLORIDE AND HYDROGEN ION DISTRIBUTIONS IN HEMOLYMPH AND MOULTING FLUID DURING THE LARVAL-PUPAL TRANSFORMATION OF BOTH FOLIAGE AND LAB REARED SILKMOTHS (Hyalophora cecropia)

I. INTRODUCTION

The silkmoth, Hyalophora cecropia (Lepidoptera, Saturniidae), is one of the most extensively studied and widely used insect models. Of particular interest is the use of the silkmoth in studies regarding ion transport by the larval midgut (Harvey and Wood, 1973; Zeranh, 1975; Blankemeyer and Harvey, 1978) and the integumentary epithelium (Jungreis and Harvey, 1975; Jungreis, 1979). Accordingly, the cation distributions in midgut (Jungreis, 1973; Jungreis and Tojo, Harvey, et al., 1973), hemolymph (Jungreis, Jatlow and Watt, 1973) and moulting fluid (Jungreis, 1974) have been analyzed. With the advent of an artificial diet (Riddiford, 1968) it became possible to raise silkmoths in the lab year, rather than on foliage of the wild cherry tree, Prunus spp., or the willow, Salix spp., during the spring and summer only. Changes in inorganic ion composition of hemolymph has been studied during development in both foliage and lab reared H. cecropia (Jungreis, Jatlow and Wyatt, 1973), but this analysis dealt primarily with changes in cation distributions (K^+, Na^+, Mg^{++}, Ca^{++}). Although chloride concentrations were also determined by these workers, electroneutrality

was not achieved, that is, a major anionic component or components was/were missing.

Studies of K^+ transport by midgut and integument have not dealt with the unknown accompanying anion (Harvey and Zerahn, 1969; Jungreis and Harvey, 1975). I undertook this study to gain a more complete picture of the anionic composition of both hemolymph and moulting fluid (see also Jungreis, 1979). Foliage and snythetic diet insects were routinely employed and I have therefore analyzed H. cecropia reared by both techniques.

Harvey and Jungreis (1975) postulated that by analogy with the labial gland (Kafatos, 1968) bicarbonate was the anion accompanying actively transported potassium across the integumentary epithelium during elaboration of moulting fluid. I therefore analysed bicarbonate levels in hemolymph and moulting fluid throughout the larval-pupal transformation. In addition I also report the levels of chloride and hydrogen ion for this period. Based on the considerable differences in bicarbonate concentrations (in both hemolymph and moulting fluid) between foliage and lab reared animals, I postulate that the natural diet confers both an enzymatic competence (interms of carbonic anhydrase) and an ability to regulate Cl^- movements that the synthetic diet does not. Therefore, the use of different rearing techniques may be an extremely useful experimental tool in the elucidation of mechanisms involved in fluid and ion transport during insect development.

II. MATERIALS AND METHODS

Lab Reared Silkmoths

Silkmoths were reared in the laboratory on the artificial diet of Riddiford (1968; personal communication) at 25°C on a light regimen of 12L:12D. Animals were staged (Jungreis, 1979) and hemolymph and moulting fluid collected (Jungreis, 1973) according to procedures reported previously.

Foliage Reared Silkmoths

Silkmoths were reared both in the laboratory and outdoors on wild cherry leaves (Prunus serotina). Animals were maintained indoors on wild cherry leaves obtained from trees around campus. Entire branches were clipped from trees, leaves rinsed thoroughly in deionized water and appropriate size stems placed in 1 to 2 liter jars filled with tap water. The jars with leaves were placed in wire mesh cages (.58 M^2 by .86 M high) in a rearing room maintained at 25°C on a photoperiod regimen of 12L:12D. Animals were taken from the field (see below) at either the 3rd or 4th instar and placed on the leaves in the lab. Foliage was changed every 2 to 4 days.

Animals were also maintained in the field (University of Tennessee Arboretum) by placing cocoons (previously held at 4-5°C for a least 6 months) inside cloth mesh nets placed over entire cherry trees. Animals were checked every two weeks throughout the spring and summer and a portion of the 3rd and 4th instar larvae removed and brought back to the lab and transported onto foliage held in the lab (see above). Mature

feeding fifth instar larvae were collected twice weekly later in the summer and brought back to the lab for staging and fluid collection. No differences were observed between animals reared entirely in the field and those reared partly in the field (up to the 3rd or 4th instar) and partly in the lab. The two groups are therefore collectively called foliage reared animals.

Analysis of Chloride and Bicarbonate

The concentration of chloride in hemolymph and moulting fluid was determined coulmetrically with a Buchler-Cotlove Chloridometer (Model 4-2008; Buchler Instruments, Fort Lee, New Jersey). Duplicate 10 or 20 ul samples were analyzed with better than ± 5% agreement between duplicate analyses.

Analysis of bicarbonate was via the micro-diffusional method of Johnston and Jungreis (1978).

Analysis of pH

The pH of moulting fluid and hemolymph was determined with a miniature combination pH electrode (1.2 mm bulb diameter, Model MI-410, Micro-electrodes Inc., Londonderry, New Hampshire) connected to a Corning Model 12 pH meter (read on expanded scale). The pH of hemolymph and moulting fluid was determined *in situ* and following collection via the method of Jungreis (1973).

III. RESULTS

Bicarbonate in Hemolymph up to the Time of Moulting Fluid Secretion in Both Foliage and Lab Reared Silkmoths

During the 8 day developmental period between mature feeding fifth larvae and 4 days after apolysis (the so-called pre-pupae of C. M. Williams) regardless of diet, hemolymph bicarbonate levels are fairly constant (around 6 to 8 mM; see Tables 3.1 and 3.2, and Figure 3.1). There is, however, a transient 1.5 fold increase that occurs 1 day after initiation of spinning (Figure 3.1) in both foliage and lab reared animals which probably reflects the substantial loss of hemolymph volume accompanying spinning of the cocoon (see Jungreis and Tojo, 1973). The volume of hemolymph decreases throughout this period of development (Table 3.3), so that the actual number of bicarbonate equivalents present at any specific stage is a more accurate measure of real changes in bicarbonate. The total number of microequivalents of bicarbonate in hemolymph from both groups of animals decreases between the late feeding fifth instar larva and the day of apolysis [46 to 18 and 46 to 24 microequivalents in foliage (Table 3.4) and lab reared silkworms (Table 3.5), respectively].

Chloride in Hemolymph up to the Time of Moulting Fluid Formation in Both Foliage and Lab Reared Silkmoths

During the first 3 days of the larval-pupal transformation in response to the decreased blood volume (Table 3.3), the levels of chloride in hemolymph of both foliage and lab reared animals increase from 12 mM (feeding fifth) to 27 mM (spin + 2 days through apolysis +

TABLE 3.1. The concentration of chloride, bicarbonate, and hydrogen ion in hemolymph and moulting fluid of foliage reared silkmoths, Hyalophora cecropia.

Stage of Development	Hemolymph			Moulting Fluid		
	mM Cl^-	mM HCO_3^-	pH	mM Cl^-	mM HCO_3^-	pH
Feeding Fifth	12.3±1.9 n=3	6.1±.42 n=3	6.83±.015 n=3			
Day of Spin	15.5±1.5 n=3	6.3±.70 n=3	6.84±.040 n=3			
Spin + 1 Day	18.6±2.3 n=4	10.2±1.0 n=4	6.76±.048 n=4			
Spin + 2 Days	26.1±3.0 n=4	6.3±1.3 n=4	6.73±.030 n=4			
Day of Apolysis	23.0±2.8 n=3	5.7±.45 n=3	6.74±.040 n=3			
Apolysis + 2 Days	26.4±2.5 n=4	6.0±1.0 n=4	6.78±.003 n=4			
Apolysis + 3 Days	21.7±.44 n=3	8.1±1.9 n=3	6.71±.016 n=3			
Apolysis + 4 Days	19.2±.68 n=3	5.3±.30 n=3	6.76±.014 n=5			
Early Moulting Fluid	23.4±1.7 n=3	14.2±3.6 n=3	6.74±.042 n=3	24.3±2.1 n=3	40.7±6.72 n=3	7.21±.029 n=3
Middle Moulting Fluid	23.3±1.6 n=3	33.3±6.5 n=3	6.69±.025 n=5	21.9±1.0 n=3	93.2±19.7 n=3	7.21±.031 n=3
Late Moulting Fluid	18.3±1.5 n=3	27.8±5.3 n=3	6.68±.034 n=4	23.8±3.6 n=3	93.1±17.0 n=3	7.09±.040 n=3

± = the standard error of the mean.

n = the number of individual observations.

TABLE 3.2. The concentration of chloride, bicarbonate, and hydrogen ion in hemolymph and moulting fluid of lab reared silkmoths, Hyalophora cecropia.

Stage of Development	Hemolymph			Moulting Fluid		
	mM Cl^-	mM HCO_3^-	pH	mM Cl^-	mM HCO_3^-	pH
Feeding Fifth	12.4±1.9 n=6	6.2±.43 n=6	6.73±.012 n=6			
Day of Spin	15.3±1.4 n=4	6.1±.42 n=4	6.77±.036 n=4			
Spin + 1 Day	18.5±2.4 n=4	11.3±1.5 n=4	6.72±.041 n=4			
Spin + 2 Days	28.0±2.8 n=3	5.0±1.2 n=3	6.71±.040 n=3			
Day of Apolysis	23.1±2.4 n=3	5.7±.47 n=3	6.67±.061 n=3			
Apolysis + 2 Days	26.2±3.2 n=4	6.3±1.3 n=4	6.72±.016 n=3			
Apolysis + 3 Days	23.5±2.6 n=3	5.0±.80 n=3	6.68±.042 n=3			
Apolysis + 4 Days	20.0±.63 n=5	7.1±1.3 n=5	6.64±.037			
Early Moulting Fluid	19.3±3.4 n=7	5.2±2.0 n=4	6.59±.029 n=8	35.2±7.0 n=7	37.9±11.5 n=4	7.21±.050 n=8
Middle Moulting Fluid	18.9±1.4 n=10	4.2±1.4 n=8	6.56±.012 n=12	31.0±2.7 n=10	36.4±12.8 n=5	7.11±.025 n=12
Late Moulting Fluid	24.6±2.3 n=9	4.8±1.6 n=4	6.54±.046 n=5	29.8±1.0 n=8	50.9±13.6 n=4	7.21±.070 n=5

± = the standard error of the mean.

n = the number of individual observations.

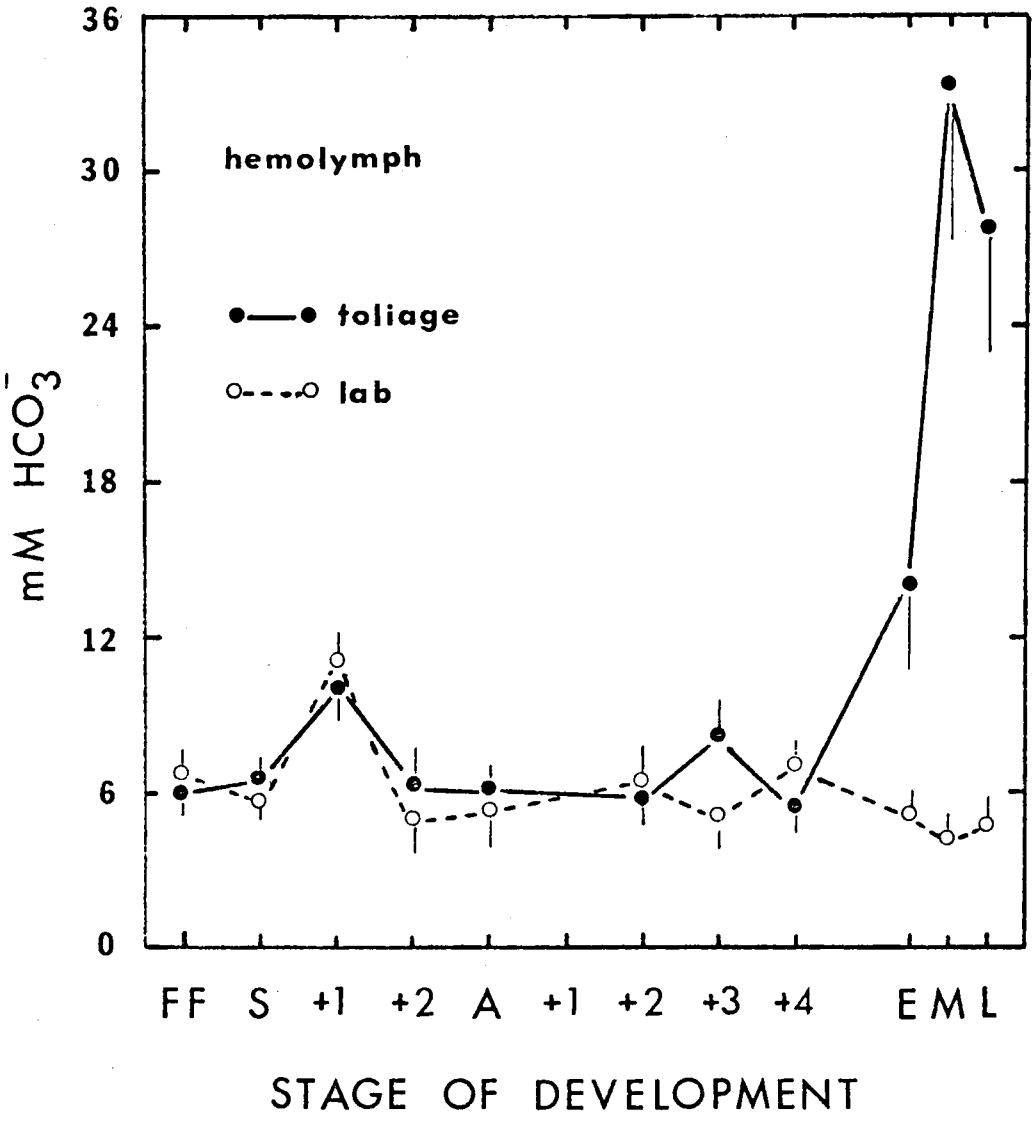


Figure 3.1. Bicarbonate concentration (mM) in hemolymph of both foliage and lab reared H. cecropia during the larval-pupal transformation.

TABLE 3.3. Volume (in milliliters) of hemolymph and moulting fluid of both foliage and lab reared H. cecropia.

Stage of Development	Hemolymph Volume	Moulting Fluid Volume
Feeding Fifth	7.5	
Day of Spin	5.6	
Day of Apolysis	3.7	
Apolysis + 2 Days	3.5	
Apolysis + 4 Days	3.4	
Early Moulting Fluid	3.4	.05
Middle Moulting Fluid	3.1	.30
Late Moulting Fluid	3.2	.15
Ecdysis	3.3	

TABLE 3.4. Total number of microequivalents of bicarbonate (A) and chloride (B) in both hemolymph and moulting fluid in foliage reared H. cecropia.

	Feeding Fifth	Day of Spin	Apolysis	Apolysis + 2 Days	Apolysis + 4 Days	Moulting Fluid Elaboration Early	Middle	Late
A				Bicarbonate Equivalents $\times 10^{-6}$				
Hemolymph	46	35	21	21	18	47	103	86
Moulting Fluid						2	28	14
Total	46	35	21	21	18	49	131	100
B				Chloride Equivalents $\times 10^{-6}$				
Hemolymph	92	87	85	91	65	76	78	56
Moulting Fluid						1	6	4
Total	92	87	85	91	65	77	84	60

TABLE 3.5. Total number of microequivalents of bicarbonate (A) and chloride (B) in both hemolymph and moulting fluid in lab reared H. cecropia.

	Feeding Fifth	Day of Spin	Apolysis	Apolysis + 2 Days	Apolysis + 4 Days	Moulting Fluid Elaboration Early	Middle	Late
A			Bicarbonate Equivalents $\times 10^{-6}$					
Hemolymph	46	34	21	22	24	17	13	15
Moulting Fluid						2	11	8
Total	46	34	21	22	24	19	24	23
B			Chloride Equivalents $\times 10^{-6}$					
Hemolymph	93	86	85	92	68	64	59	76
Moulting Fluid						2	9	4
Total	93	86	85	92	68	66	68	80

2 days; Tables 3.1-3.2, pp. 46-47, and Figure 3.2). Between apolysis + 2 days, until the initiation of moulting fluid formation (3 to 3-1/2 days later) the chloride levels are noted to decrease (26 mM at apolysis + 2 days to 19 mM at Warly Moulting Fluid, Tables 3.1-1.2 and Figure 3.2). Although the chloride concentration increases during the first 3 days of the larval-pupal transformation, the total number of chloride microequivalents (85 to 93) is invariant through apolysis + 2 days, but drops sharply by apolysis + 3 days to 65-68 microequivalents (Tables 3.4 and 3.5).

Hemolymph Bicarbonate During Moulting Fluid Elaboration in Foliage Reared Silkmoths

As foliage reared H. cecropia begin the process of moulting fluid elaboration, secretion and resorption at 8.5 days after spinning or 5.5 days after apolysis, there is a significant increase in the concentration of hemolymph bicarbonate (5.3 mM at apolysis + 4 days to 14 mM at early moulting fluid, $p < 0.05$, see Table 3.1). This increase continues through the middle and late moulting fluid stages (12 and 24 hours later) until hemolymph bicarbonate has increased more than 6 fold (5.3 mM at apolysis + 4 days and 33 mM at middle moulting fluid, $p < 0.01$, see Table 3.1 and Figure 3.1). Although the hemolymph volume continues to decline during this period, the decreased volume is not responsible for the 6 fold increase in hemolymph bicarbonate (Table 3.4). During resorption of moulting fluid (= late moulting fluid), bicarbonate levels begin to decline (35 mM at middle to 27 mM at late, Table 3.1, Figure 3.1; or 103 microequivalents at middle to 86 microequivalents at late,

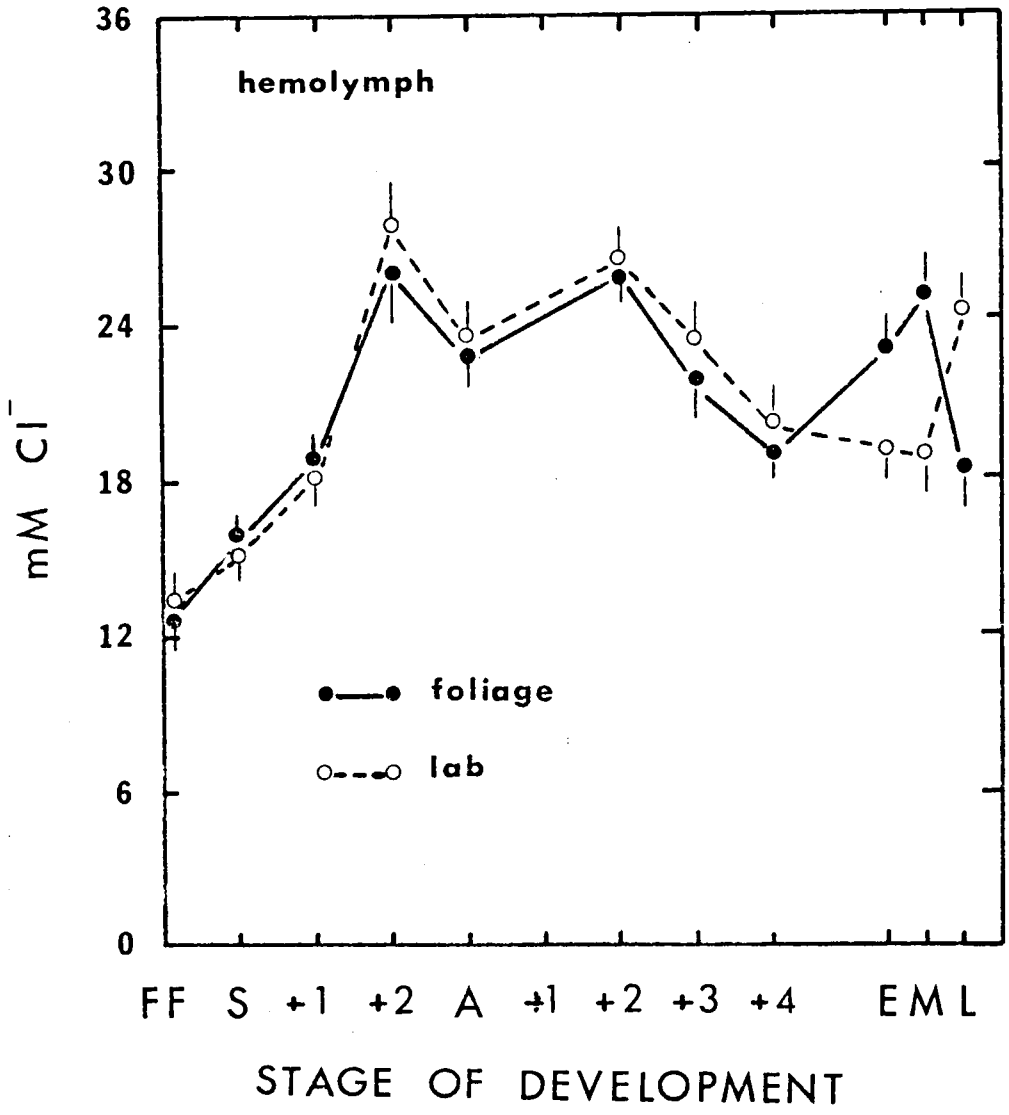


Figure 3.2. Chloride concentration (mM) in hemolymph of both foliage and lab reared H. cecropia during the larval-pupal transformation.

Table 3.4) until levels of 6 to 8 mM are restored in the newly ecdysed pupa (data not shown).

Hemolymph Bicarbonate During Moulting Fluid Elaboration in Lab Reared Silkmoths

Lab reared H. cecropia do not exhibit the dramatic increases in hemolymph bicarbonate characteristic of foliage reared animals. During elaboration of moulting fluid, the concentration of bicarbonate in hemolymph declines in diet reared silkmoths (7 mM at apolysis + 4 days to 4 mM at middle moulting fluid; Table 3.2, p. 47, and Figure 3.1, p. 48). This decline reflects a decrease in the absolute number of bicarbonate microequivalents present (24 microequivalents at apolysis + 4 days but only 13 microequivalents at middle moulting fluid, $p < 0.01$; Table 3.5).

Hemolymph Chloride in Foliage and Lab Reared Animals During Moulting Fluid Elaboration

In foliage reared silkmoths, hemolymph chloride levels increase during the first stages of moulting fluid secretion (19 mM at apolysis + 4 days to 25 mM at middle moulting fluid; Table 3.1, p. 46, and Figure 3.2). During periods of moulting fluid resorption (i.e., late moulting fluid) the levels of chloride in hemolymph drop to 18 mM (Table 3.1, Figure 3.2).

In lab reared silkmoths these trends are not observed. Hemolymph chloride levels do not increase during periods of moulting fluid elaboration, despite the fact that lab reared animals produce the same volume of moulting fluid as do foliage reared animals (Table 3.3, p. 49).

Chloride levels remain around 18-20 mM during the periods of early and middle moulting fluid (Table 3.2, p. 47; Figure 3.2), increasing only in the later phases of moulting fluid resorption (up to 25 mM, Table 3.2).

pH of Hemolymph in Foliage and Lab Reared Silkmoths

The pH of hemolymph in foliage reared H. cecropia is 6.8 at the initiation of spinning (Table 3.1, p. 46; Figure 3.3). After the outer cocoon has been completed, the pH falls to around 6.75 and is maintained at this level until middle and late moulting fluid elaboration, when the pH drops further to around 6.70 (Table 3.1, Figure 3.3).

In lab reared animals the stage specific pH of hemolymph is always more acidic than in foliage reared animals (Figure 3.3). The pH of hemolymph is 6.77 at the initiation of spinning and 6.72 at apolysis + 2 days, but falls dramatically to a low of 6.54 ($p < 0.05$) during resorption of moulting fluid (Figure 3.3).

Bicarbonate in Moulting Fluid of Foliage Reared Silkmoths

Moulting fluid bicarbonate levels in foliage reared animals increases from 40 mM (early) to 93 mM (middle and late; Table 3.1, Figure 3.4). While lab reared animals have equivalent concentrations during the early phases (40 mM; see Table 3.2, Figure 3.4), only a slight increase in bicarbonate concentration is noted during the later phases of moulting fluid resorption (50 mM in lab but 93 mM in foliage reared animals; Tables 3.1 and 3.2, Figure 3.4).

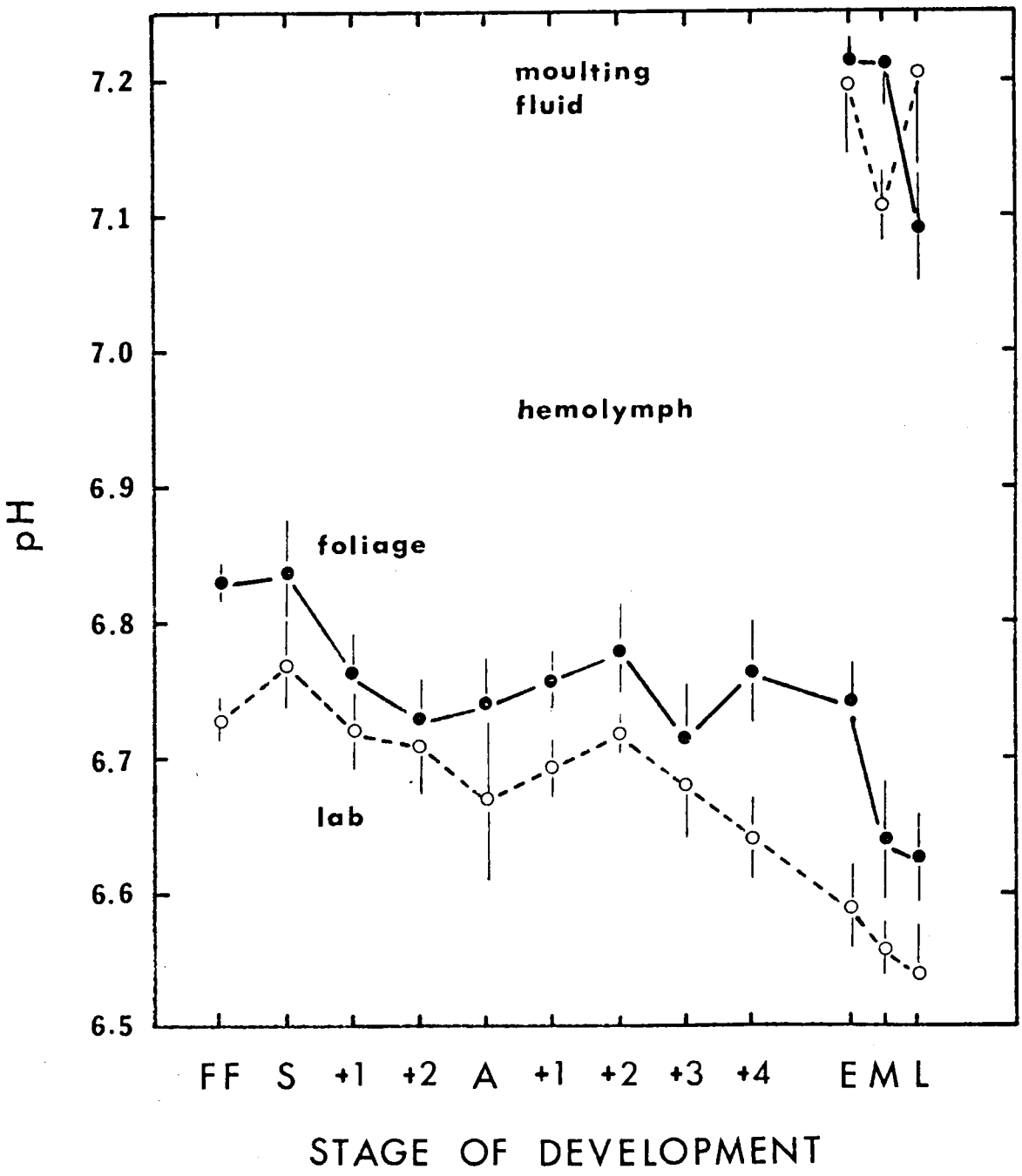


Figure 3.3. pH of hemolymph and moulting fluid during the larval-pupal transformation of both foliage and lab reared H. cecropia.

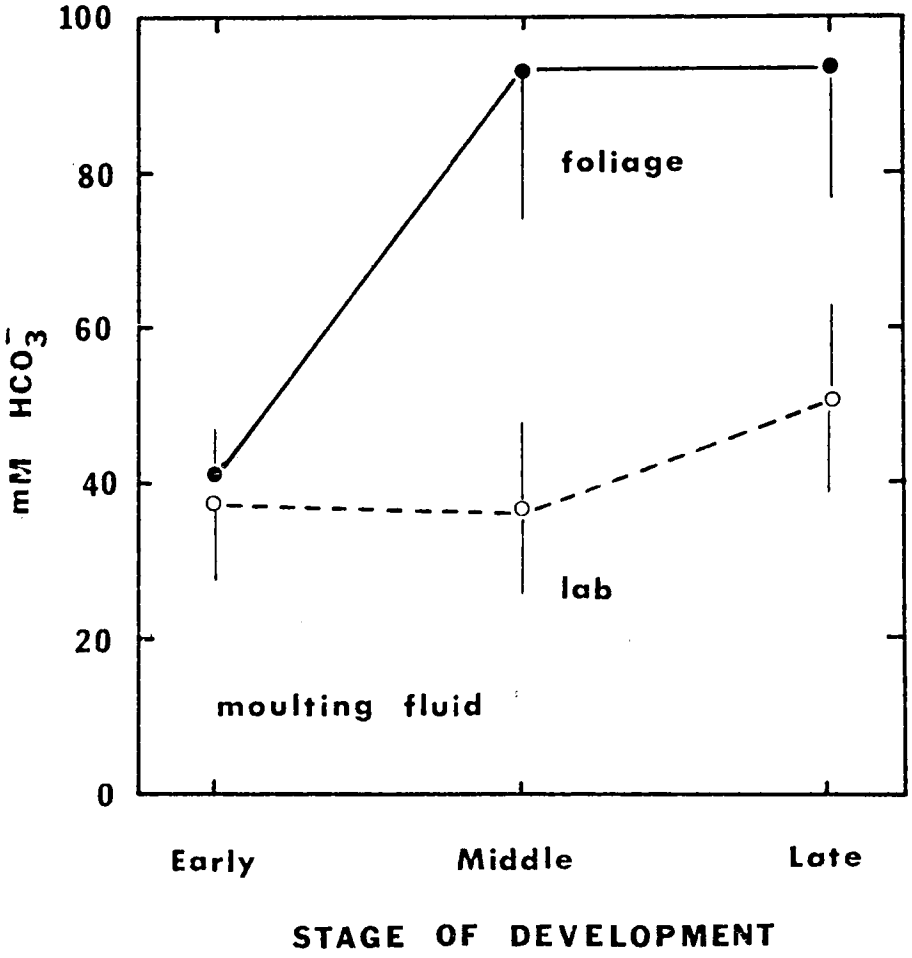


Figure 3.4. Bicarbonate concentration (mM) in moulting fluid during early, middle and late stages of moulting fluid secretion and resorption in both foliage and lab reared H. cecropia.

Foliage reared silkmoths have 3 to 5 times higher concentration ratios of bicarbonate present in moulting fluid than in hemolymph (Table 3.6), whereas lab reared animals have only twice as much bicarbonate in moulting versus hemolymph (Table 3.7).

Chloride in Moulting Fluid of Foliage and Lab Reared Silkmoths

Foliage reared H. cecropia have 21-24 mM chloride in moulting fluid contrasted with 18-25 mM chloride in hemolymph (Table 3.1, p. 46). The moulting fluid/hemolymph ratio for chloride is approximately 1.0. Lab reared silkmoths have 30% higher concentrations of chloride in moulting fluid than do foliage reared animals (Tables 3.1 and 3.2, pp. 46-47; Figure 3.5). The concentration of chloride in moulting fluid is 30-35 mM, while that in hemolymph is 19-25 mM (Table 3.2). Lab reared animals have 1.2 to 1.8 times as much chloride in moulting fluid as in hemolymph (Table 3.7).

pH of Moulting Fluid in Foliage and Lab Reared Silkmoths

Moulting fluid in both foliage and lab reared animals is a slightly basic solution. Initially (during early moulting fluid secretion), the pH is 7.2 regardless of rearing technique. In foliage reared animals the pH is maintained at 7.2 through the middle periods of moulting fluid secretion and then falls to 7.09 during moulting fluid resorption. Lab reared animals differ from foliage animals in that the pH of their moulting fluid falls to 7.11 in middle moulting fluid, but then rises to the initial level of 7.21 during late moulting fluid resorption (Tables 3.1 and 3.2, Figure 3.3).

TABLE 3.6. The moulting fluid/hemolymph ratio for bicarbonate (A) and chloride (B) in foliage reared H. cecropia during early, middle, and late moulting fluid stages.

	Moulting Fluid Stages		
	Early	Middle	Late
A		Bicarbonate	
Moulting Fluid / Hemolymph	1.77±.084 n=3	3.68±1.62 n=3	5.09±1.71 n=3
B		Chloride	
Moulting Fluid / Hemolymph	1.04	.83±.17 n=3	1.30±.28 n=3

± = the standard error of the mean.

n = the number of paired data.

TABLE 3.7. The moulting fluid/hemolymph ratio for bicarbonate (A) and chloride (B) in lab reared H. cecropia during early, middle, and late moulting fluid stages.

	Moulting Fluid Stages		
	Early	Middle	Late
A		Bicarbonate	
Moulting Fluid / Hemolymph	1.96±.61 n=4	1.93±.72 n=5	2.07±.84 n=4
B		Chloride	
Moulting Fluid / Hemolymph	1.82±.63 n=4	1.64±.21 n=10	1.21±.36 n=8

± = the standard error of the mean.

n = the number of paired data.

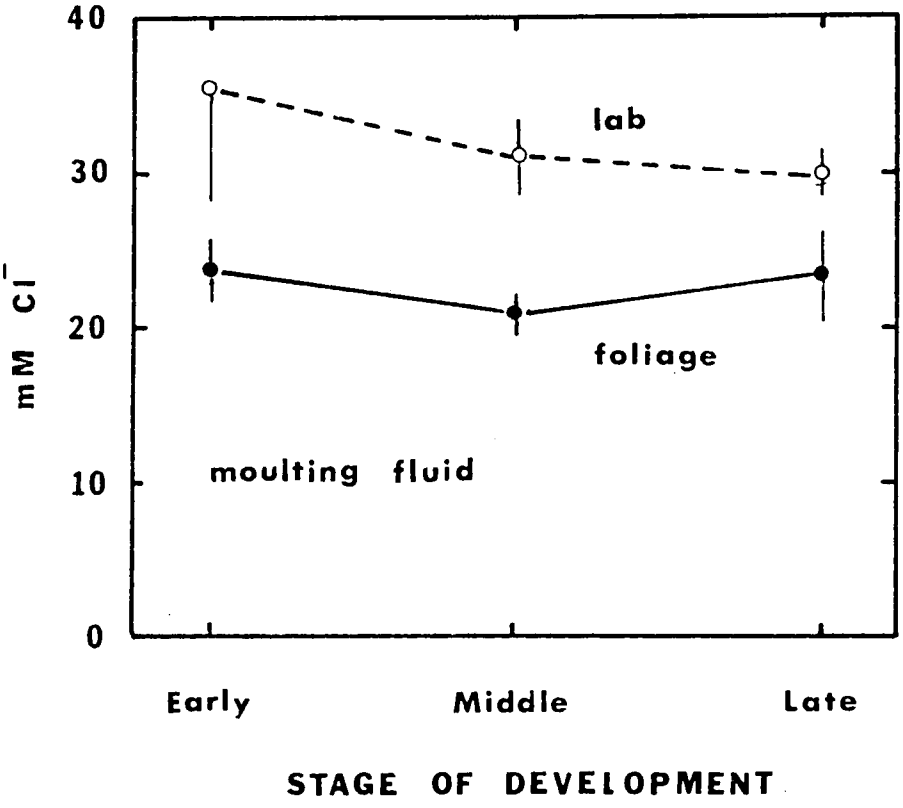


Figure 3.5. Chloride concentration (mM) in moulting fluid during early, middle and late stages of moulting fluid secretion and resorption in both foliage and lab reared H. cecropia.

IV. DISCUSSION

Bicarbonate and Chloride in Hemolymph Prior to Moulting Fluid Elaboration

During the first 8 days of the larval-pupal transformation the concentration of HCO_3^- in hemolymph of both foliage and lab reared animals remains relatively constant around 6 mM except for a single peak of increased concentration at spin + 1 day. This level of hemolymph bicarbonate is in general agreement with bicarbonate levels reported in other species (most levels reported are between 2 and 15 mM, see Bishop, 1953; Buck, 1953; Demjanowski, et al., 1932, Levenbook, 1950; Jungreis, 1979). This constancy of bicarbonate concentration in H. cecropia hemolymph is maintained in the face of a 50% decline in fluid volume over this period. I attribute the decreased number of bicarbonate equivalents to a decrease in the enzymatic capacity (- 50%) to synthesize bicarbonate. Total carbonic anhydrase (E.C. 4.2.1.1) present in midgut, fat body and integumentary epithelium decreases 50% during this period (see Chapter 5).

The increase in bicarbonate concentration seen at the initiation of spinning is due entirely to fluid losses associated with formation of the cocoon (volumes of 7.5 ml before and 3.7 ml after, Table 3.3, p. 49). After the cocoon in spun, the loss of hemolymph HCO_3^- is associated with a decrease in carbonic anhydrase activity. Thus the levels of HCO_3^- in hemolymph return to pre-spinning levels in both foliage and lab reared animals.

Fluid losses during the first 8 days of the larval-pupal transformation are responsible for increases in both the concentrations

of hemolymph chloride and bicarbonate, although the peak for chloride is 24 hours out of phase (later) with bicarbonate indicating a continued movement of chloride from some non-hemolymph pool (Figures 3.1, p. 48, and 3.2, p. 53). Chloride remains relatively constant in both the leaf and artificial diet silkworms until apolysis + 2 days, when chloride begins to move out of hemolymph and into the cells. This phenomenon occurs in both foliage and lab reared animals (Figure 3.2).

The relationship between bicarbonate and chloride in hemolymph is such that, although the concentrations of the components fluctuate sharply prior to moulting fluid secretion, chloride represents a constant percentage (average equals 75%) of the total HCO_3^- + Cl^- (Figure 3.6). For example, in foliage reared animals at apolysis + 2 days hemolymph chloride is 26 mM or 81% of the HCO_3^- + Cl^- total while at apolysis + 4 days the hemolymph chloride concentration is only 19 mM, yet this represents 79% of the total.

Bicarbonate and Chloride in Hemolymph During Moulting Fluid Formation

Prior to moulting fluid elaboration both foliage and lab reared silkmoths exhibit comparable patterns of hemolymph bicarbonate and chloride regulation. Thereafter, rather marked and important differences develop between animals reared on the different diets. A 6 fold increase in hemolymph bicarbonate occurs in foliage animals from apolysis + 4 days to middle moulting fluid (5 mM to 33 mM), whereas bicarbonate levels in lab reared animals actually decline (7 mM to 4 mM, Figures 3.2 and 3.7). These differences are not the result of

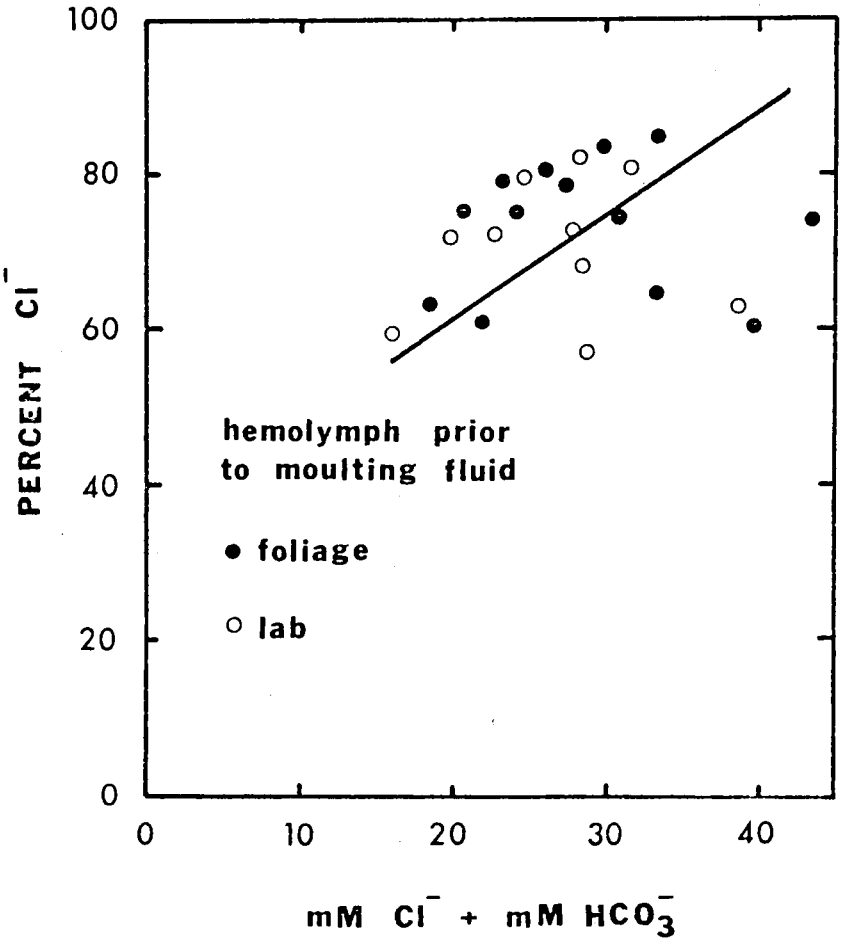


Figure 3.6. Relationship between the percentage of chloride and the total bicarbonate-chloride concentration in hemolymph prior to secretion of moulting fluid. There is no significant difference ($p > 0.05$) between foliage and lab reared groups.

differential moulting fluid volumes, nor of differential declines in the enzymatic capacities to produce bicarbonate (in vitro) per se (see Chapter 5). Rather, differences can be attributed to two factors, both of which are strongly influenced by chloride. First, I have shown that in the absence of Cl^- carbonic anhydrase in fat body and integument from lab reared animals is capable of producing bicarbonate at rates comparable to those carbonic anhydrases from foliage animals (Chapter 5). In the presence of physiological concentrations of chloride, carbonic anhydrases from lab reared animals produce bicarbonate at significantly lower rates than the comparable foliage enzymes. Secondly, during the time of moulting fluid secretion intracellular chloride is significantly higher in lab than in foliage animals. Lab animals have a reduced ability to regulate cellular chloride levels as evidenced by lower chloride titers in hemolymp (Figure 3.2, p. 53). Thus at a time when the chloride sensitive carbonic anhydrase can least tolerate high chloride, lab animals have more chloride present. It is obvious that in foliage reared animals carbonic anhydrase activity is "turned on" between apolysis + 4 days and early moulting fluid. While the cause of this "turn on" is not certain, I feel that it is related to the movement of chloride out of the cells and into hemolymph (i.e., away from the cytoplasmic compartment containing carbonic anhydrase). The key point is that this chloride movement does not occur in lab reared animals, causing a significant inhibition of integumentary carbonic anhydrase, resulting in a reduced capacity to produce high levels of bicarbonate. Evidence for this chloride movement is that hemolymph chloride

equivalents increase by 15% in foliage reared animals only between apolysis + 4 days and middle moulting fluid. The additional chloride must come from non-hemolymph sources since no dietary input has occurred for 8 days. This trend is reversed during late moulting fluid, a period in which the levels of hemolymph chloride fall sharply (- 30%) in foliage reared animals. Resorption of moulting fluid per se, is unrelated to this decline, since the increase in hemolymph volume attributable to moulting fluid resorption would amount to only 3%. Further, moulting fluid is equimolar or higher in chloride concentration relative to hemolymph, so that resorption of this fluid would certainly not cause a decrease in hemolymph chloride levels. I believe that this decrease is caused by a reversal of the chloride efflux (cells $\longrightarrow$ hemolymph) seen during early moulting fluid periods. I propose that during moulting fluid resorption hemolymph chloride returns to some non-hemolymph pool. It is worth mentioning that carbonic anhydrase activity in foliage reared animals falls markedly during resorption of moulting fluid (Chapter 5).

In lab reared animals similar trends are not observed. Chloride levels remain relatively constant throughout early and middle moulting fluid (18-20 mM) rising only during the later stages of moulting fluid resorption. These trends are opposite those seen in similarly staged foliage animals (see Figures 3.2, p. 53, and 3.7).

In summary, during periods of moulting fluid production lab reared animals are unable to produce bicarbonate <u>in vivo</u> at rates comparable to those seen in foliage animals. Secondly, in foliage animals only,

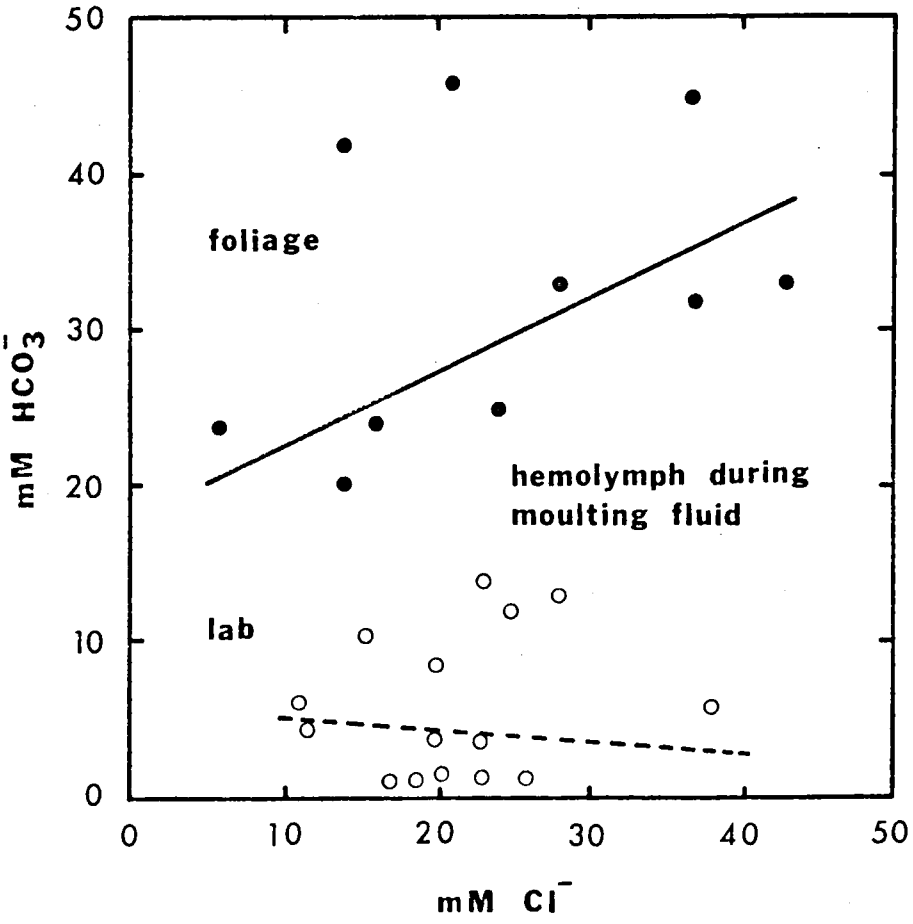


Figure 3.7. Relationship between hemolymph bicarbonate (mM) and chloride (mM) concentrations during stages of moulting fluid secretion and resorption in foliage and lab reared H. cecropia.

chloride accumulates in hemolymph during early and middle moulting fluid and is lost from hemolymph during resorption of moulting fluid. Chloride reduces the enzymatic capacity of carbonic anhydrase within the insect's tissues (to a much greater extent in diet than foliage reared animals, Chapter 5), and the observed movement of chloride into and out of hemolymph reflects a putative role in the regulation and production of bicarbonate.

Bicarbonate in Moulting Fluid

Foliage reared H. cecropia have 2.5 times more bicarbonate in moulting fluid than do diet reared animals (Tables 3.1 and 3.2, pp. 46-47; Figure 3.4, p. 57). I believe that the bicarbonate appearing in moulting fluid originates de novo from the integumentary epithelium rather than from hemolymph sources, since integumentary epitelium at the time of moulting fluid elaboration contains more than 50% of the whole body carbonic anhydrases present under both rearing conditions (Chapter 5). If bicarbonate appearing in moulting fluid were derived from hemolymph then in diet reared silkworms the entire quantity of bicarbonate in hemolymph (11 to 13 microequivalents; Table 3.5, p. 51) would have to pass across the integument without a compensatory rise in hemolymph HCO_3^- concentration. I believe that regulation of carbonic anhydrase in integumentary epithelium is the source of the "problem" in the inability of lab reared animals to produce high levels of bicarbonate in moulting fluid, just as chloride inhibition of fat body carbonic anhydrase is the reason why low hemolymph bicarbonate levels are observed during elaboration of moulting fluid.

High levels of bicarbonate present in moulting fluid of foliage reared silkmoths serve to provide electroneutrality for actively transported K^+ (from hemolymph to moulting fluid, Jungreis and Harvey, 1975; Jungreis, 1979) and to aid in buffering the moulting fluid so that hydrolytic enzymes are maintained within physiologically optimum pH ranges (Passonneau and Williams, 1953; Katzenellenbogen and Kafatos, 1970). In lab reared animals, bicarbonate is not produced in sufficient quantities to fully balance actively transported K^+, and chloride then accompanies the K^+ into moulting fluid (Figure 3.8).

Chloride in Moulting Fluid

Diet reared animals tend to have higher levels of chloride in moulting fluid (30% more) than do foliage reared animals. Under foliage reared conditions silkmoths have approximately equimolar concentrations of chloride in hemolymph and moulting fluid (Table 3.6, p. 59) while lab reared animals have twice as much chloride (on a molar basis) in moulting fluid than in hemolymph (Table 3.7, p. 60). In response to the absence of a dietary component present in foliage but not in the synthetic diet, lab reared animals are unable to move chloride out of the integumentary epithelial cells prior to moulting fluid formation (Figure 3.2, p. 53). The residual putative high levels of intracellular chloride inhibit carbonic anhydrase (the lab enzyme being uniquely more susceptible to chloride inhibition than the foliage enzyme) so that lower levels of bicarbonate are produced. Furthermore, during periods when K^+ is actively transported across the integument at rates which are equal in both foliage and lab reared animals, a potential is established

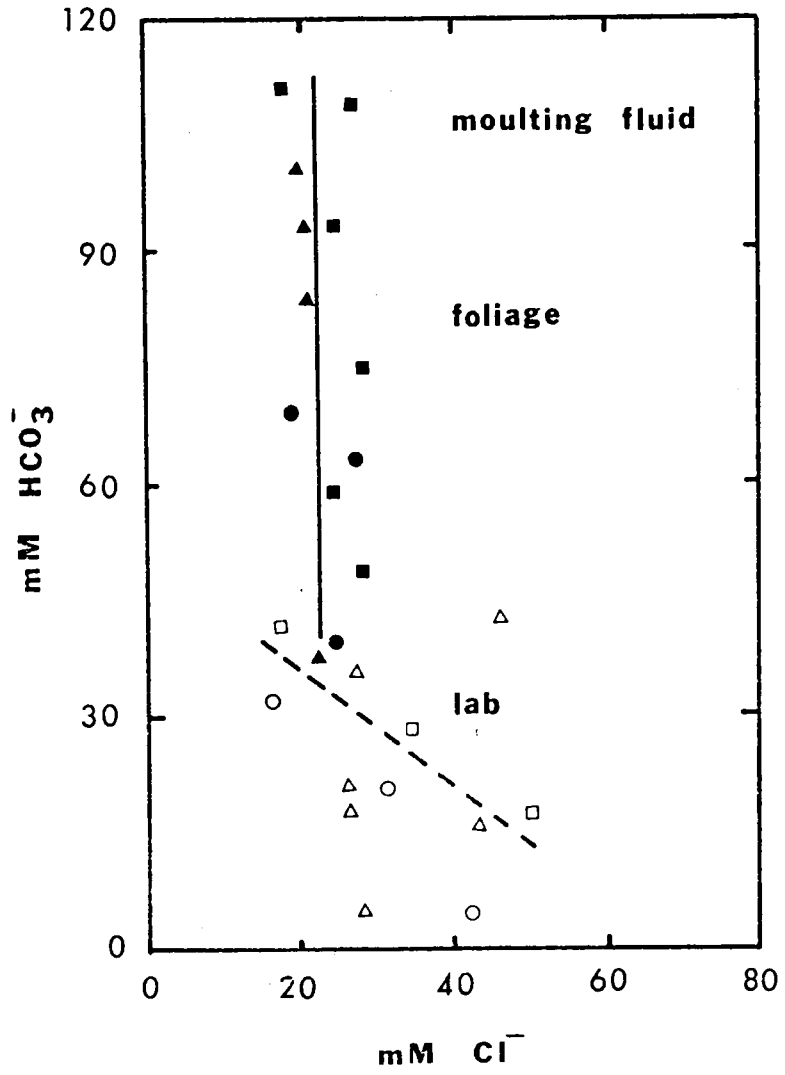


Figure 3.8. Relationship between bicarbonate and chloride in moulting fluid of both foliage and lab reared H. cecropia.

across the epidermal (exuvial side being about 10 mV positive with respect to hemolymph; see Jungreis and Harvey, 1975). This potential enhances the movement of intracellular chloride in lab reared animals into the exuvial space (i.e., into moulting fluid). Chloride thus serves to substitute for bicarbonate during K^+ transport.

Although silkmoths raised on artificial diet are different from foliage reared animals, they have been able to adapt sufficiently such that the larval-pupal transformation is successfully completed as often in lab as in foliage reared animals. This adaptative capacity of diet reared integument should be studied more intensively so as to gain insight into the role of ion transport (namely, HCO_3^-, Cl^- and K^+) during insect moulting. This approach to insect rearing (i.e., different diets) opens the door to further study involving control of compartmentalization of potentially important substances responsible for controlling the moult. And lastly, this experimental approach can provide invaluable insight into how enzymes are regulated during insect growth and metamorphosis.

CHAPTER 4

CARBONIC ANHYDRASE DURING THE LARVAL-PUPAL TRANSFORMATION OF THE TOBACCO HORNWORM (Manduca sexta): EFFECTS OF POTASSIUM AND CHLORIDE ON MIDGUT, FAT BODY AND INTEGUMENTARY ENZYMES

I. INTRODUCTION

The processes underlying ecdysis are poorly understood. Periodic ecdyses enable an insect to grow, and an understanding of this process is of key importance in elucidating new methods for the control of insect vectors and pests. Although the hormonal control, the protein and nucleic acid metabolism, and the cuticular morphology of moulting have been studied (see literature citations in Jungreis, 1979), little attention has been paid to these processes whereby moulting fluid is secreted and resorbed. Studies dealing with the selective hydrolysis of the old cuticle preliminary to ecdysis have been published (katzenellenbogen and Kafatos, 1970, 1971; Jungreis, 1973, 1974; Locke and Krishnan, 1973; Bade, 1974, 1975, 1978; Bade and Shoukimas, 1974; Jungreis and Harvey, 1975; Locke, 1974, 1976; Hikida and Jungreis, 1978), but as a group these fail to provide insight into the moult process proper.

In an analysis of the components of larval-pupal moulting fluid in the tobacco hornworm, Manduca sexta, the major anionic component was found to be bicarbonate (Chapter 2; Jungreis, 1979) I now report that

this bicarbonate is produced via integumentary carbonic anhydrases. Further, having also evaluated carbonic anhydrases present in midgut and fat body, and having compared the effects of acetazolamide, potassium and chloride on these enzymes with that of carbonic anhydrase associated with the integument, I conclude that each tissue possesses a unique form of the enzyme.

Previous reports on insect carbonic anhydrases have dealt primarily with the absence of this enzyme in hemolymph, a situation which gives the insect a unique respiratory system (Buck, 1958; Buck and Friedman, 1958; Levenbook, 1950; Levenbook and Clark, 1950; Levy and Schneiderman, 1958). Only a few studies on carbonic anhydrase present in other tissues or fluid have been reported (see, for instance, Turbeck and Foder, 1970). Other studies have used techniques (acetazolamide or sodium sulfide inhibition of short circuit currents, see Haskell, et al., 1965; Maddrell, 1971; Williams, et al., 1978) which tend to support the notion that anhydrase is indeed present in some, but not all insect tissues. However, no study to date has characterized the enzyme in fat body or integument during an extended developmental period. It is hoped that, by understanding the properties of, and changes in, various tissue carbonic anhydrases during an extended portion of insect metamorphosis, important questions concerning not only insect development (see Chapter 7), but also other carbonic anhydrases (Johnston and Jungreis, 1979), can be answered.

II. MATERIALS AND METHODS

Tobacco hornworms used in these experiments were obtained from an inbred colony maintained in our laboratory. Larvae were reared on the wheat germ-casein diet of Bell and Joachim (1976) under an 18L:6D photoperiod regimen at 23 to 25°C. Animals were staged (Jungreis, 1979) and hemolymph and moulting fluid collected according to the method of Jungreis (1978).

Carbonic Anhydrase Activity

Total carbonic anhydrase activity was determined from individual animals in midgut, fat body, integument (with accompanying muscle), muscle, cuticle, hemolymph, moulting fluid and gut contents throughout each stage of the larval-pupal transformation. Individual tissues were dissected over ice, blotted, weighed and added to 5 ml of cold (4°C) 15 mM HEPES Buffer (N-2-hydroxyehtly piperazine-N'-2-ethanesulfonic acid, Sigma Chemical Co.; the pH was adjusted to 8.35 ± .02 with 1N NaOH). This tissue-buffer mixture was then sonicated at 4°C for 4 times 20 second intervals with a Brownwell Biosonik Tissue Sonicator (Brownwell Scientific, Rochester, New York). Sonicates were then centrifuged for 8 minutes in a Servall Angle Centrifuge at 4,000 × g. The carbonic anhydrase activity was measured in a 500 microliter aliquot of the supernatant (virtually 100% of the carbonic anhydrase activity is present in the supernatant fraction) via the electrometric method of Wilbur and Anderson (1948). By this method, one determines the time required (in seconds) to lower the pH of a CO_2 saturated (30.6 mM)

solution in 15 mM HEPES Buffer at 2°C from 8.3 to 6.8. A standard curve employing 0.02 to 2.15 units of Bovine carbonic anhydrase (Sigma Chemical Co.) was simultaneously determined.

Effects of K^+, Cl^- and Acetazolamide

The effects of chloride (as choline chloride) and potassium (as potassium chloride) on carbonic anhydrase activity were determined at two stages in development: feeding fifth instar, and middle moulting fluid, for midgut, fat body and integument. Activity was determined as previously described, but with the addition of 5 to 200 mM (final) concentrations of either potassium chloride (= KCl) or choline chloride (= ChCl).

The effect of acetazolamide (Sigma Chemical Co.), a known specific inhibitor of carbonic anhydrase (see Maren, 1971), was determined on the activity of the enzyme in midgut, fat body and integument from feeding fifth instar animals. The sulfonamide (10^{-9} to 10^{-5} M) was added to the final incubation mixture.

III. RESULTS

Tissue Carbonic Anhydrase Levels

The total number of carbonic anhydrase units per animal, as well as the relative portion contributed by midgut, fat body and integument are shown in Figure 4.1. Activities ranged from a high of 9 units/animal during the feeding fifth stage, to a low of less than 3 units/animal at pink stripe + 3 days (i.e., 12 hours before initiation of moulting fluid secretion). Within the next 36 hours, however, the number of carbonic

Figure 4.1. The total number of carbonic anhydrase units per animal per tissue during each stage of development in the larval-pupal transformation of M. sexta. FF = feeding fifth; PS = Pink stripe; 1,2,3, = days after the pink stripe stage; E,M,L = early, middle and late moulting fluid stages; P = pupae.

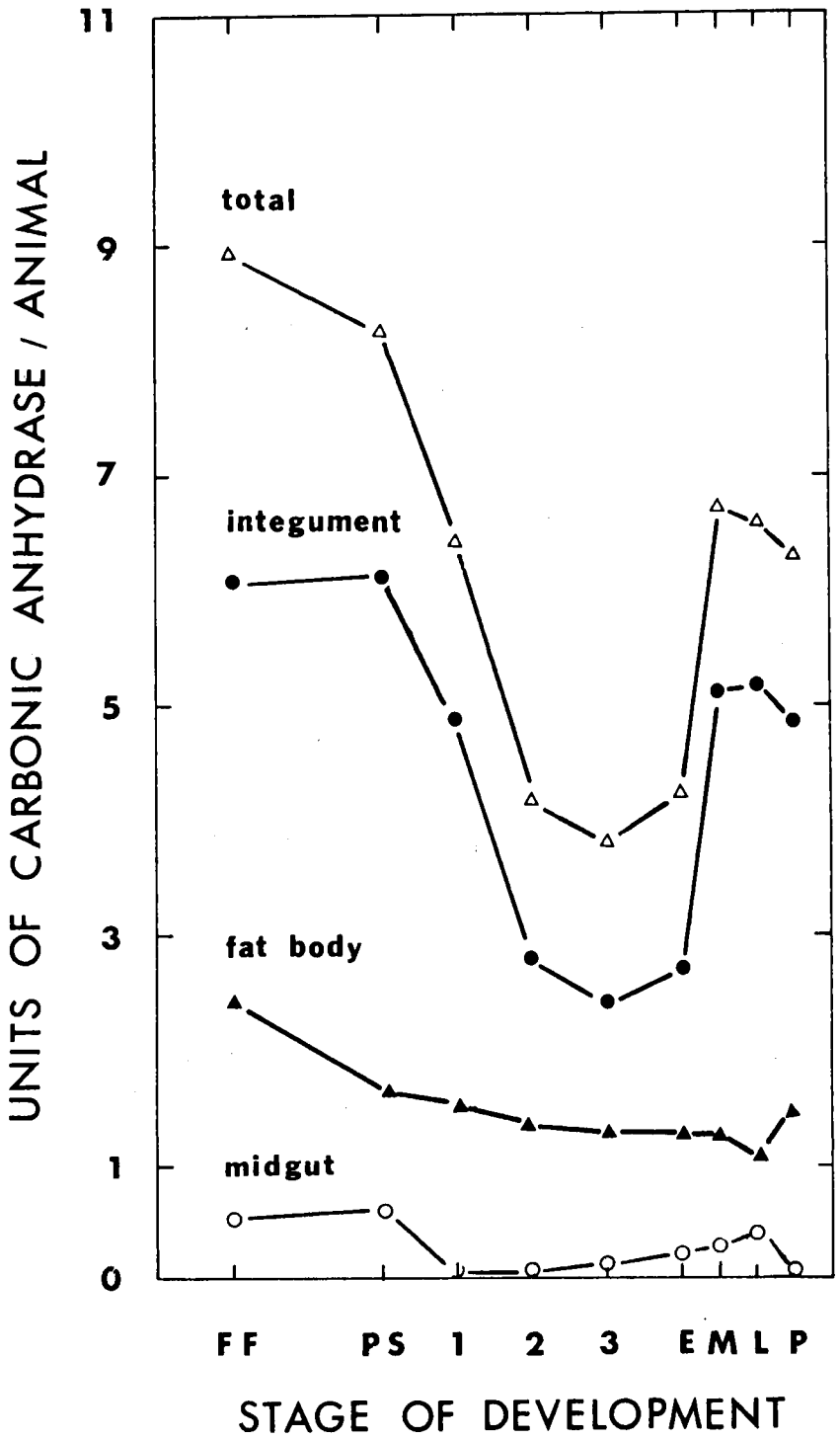

11
9
7
5
3
1
0
UNITS OF CARBONIC ANHYDRASE / ANIMAL
total
integument
fat body
midgut
FF
PS
1
2
3
E
M
L
P
STAGE OF DEVELOPMENT

Figure 4.1

anhydrase units more than doubles to nearly 7 units/animal. This pre-ecdysis increase in total body carbonic anhydrase results from an increase in activity associated with integument. At the time of moulting fluid secretion (on pink stripe + 4 days) integumentary carbonic anhydrase accounts for 70 to 80% of the total activity in the animal (Figure 4.2). The carbonic anhydrase present in the integument is not derived from associated musculature since none could be detected in the integumentary musculature at any stage in the larval-pupal development. The number of integumentary carbonic anhydrase units present during elaboration of moulting fluid (Figure 4.1) is so great as to produce, at 2°C, the total number of bicarbonate equivalents present in moulting fluid every 25 minutes (assuming a CO_2 concentration of 30.6 mMO.

Carbonic anhydrase activity could not be detected in hemolymph, muscle, larval cuticle, pupal cuticle or gut contents at any stage measured. The carbonic anhydrase activity associated with midgut does not represent total digestive tract carbonic anhydrase, since the hindgut-rectum portion of the gut is not included. In addition, integumentary carbonic anhydrase is defined as that activity associated with tissue lying exterior to the integumentary musculature and interior to the endocuticle. Furthermore, it was impossible to completely eliminate tracheal and connective tissue contamination from the tissues, especially fat body and integument.

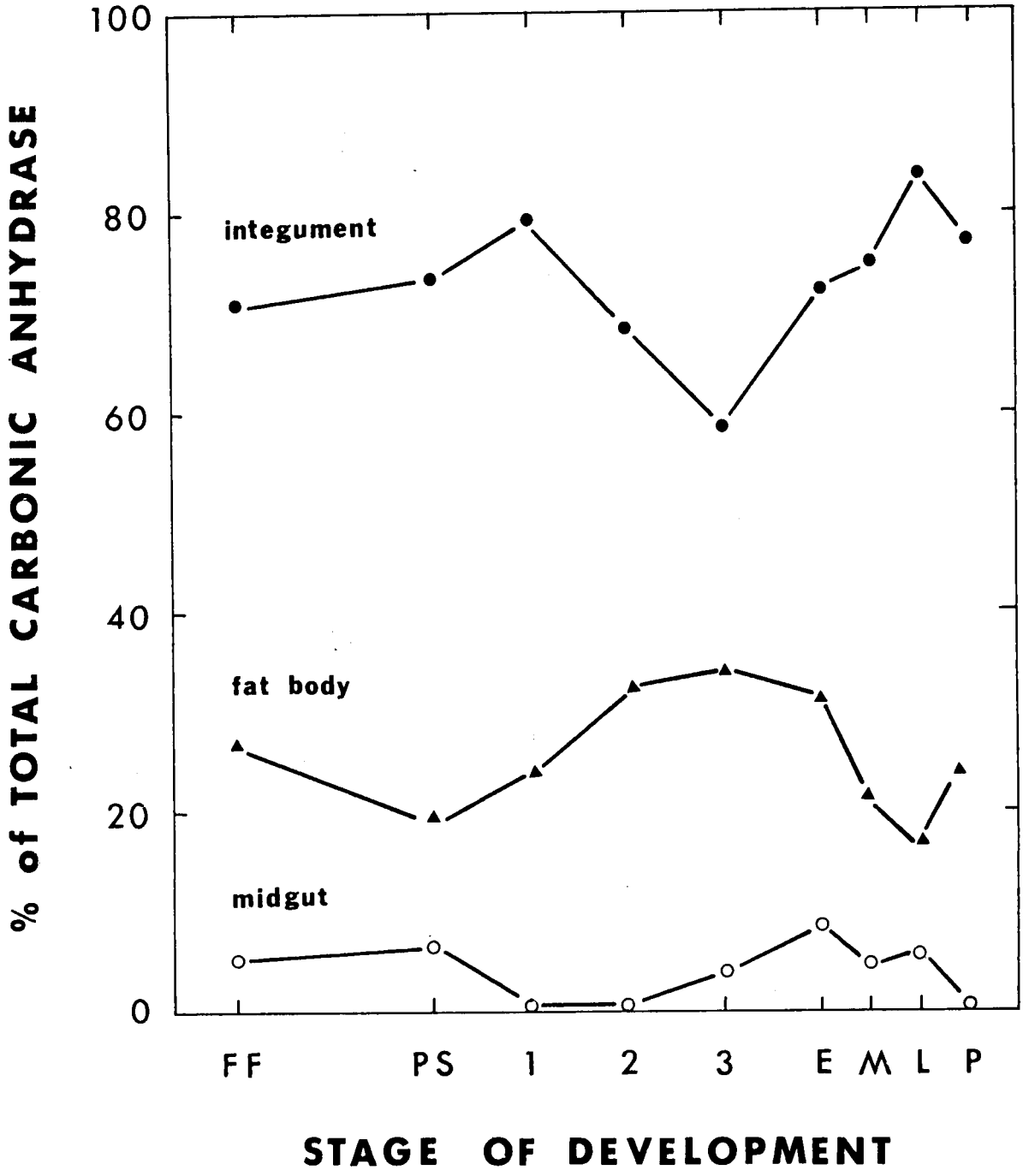


Figure 4.2. The percentage of the total carbonic anhydrase/ animal contributed by integument, fat body and midgut throughout each stage of the larval-pupal transformation in M. sexta. See Figure 4.1 for stages of development.

Effects of Acetazolamide, K^+ and Cl^- on Carbonic Anhydrase Activity

The effects of acetazolamide (10^{-9} to 10^{-5} M) on midgut, fat body and integument carbonic anhydrases were determined in feeding fifth instar hornworms (Figure 4.3). The calculated I_{50}'s (concentration of inhibitor which will cause a 50% reduction in activity) of acetazolamide for fat body is 10^{-7} M, while that for midgut and integument is 2×10^{-8} M. Mammalian carbonic anhydrases have I_{50}'s for acetazolamide in the range of about 3×10^{-8} M (Wistrand and Rao, 1968).

These differences (10^{-7} versus 2×10^{-8}) suggested the presence of tissue specific carbonic anhydrases in M. sexta. This hypothesis was tested by studying the effects of KCl and ChCl on carbonic anhydrases obtained from the 3 tissue sources. The molecular size of choline is assumed to be of such magnitude as to effectively exclude it from interacting with the active or catalytic sites. The effects of ChCl were therefore attributed to chloride alone. In a like manner, addition of KCl was interpreted as a K^+ effect superimposed upon a Cl^- effect.

K^+ appears to stimulate the enzyme (e.g., feeding fifth instar) in midgut (maximum of 60% at 30 mM or above), while Cl^- is without effect at concentrations below 100 mM (Figure 4.4). The same study done on fat body reveals that K^+ activates fifth instar enzyme (+ 50% at 10 + mM), while Cl^- is somewhat inhibitory (about - 25% t 10 to 50 mM) at both the feeding fifth and middle moulting fluid stages in development (Figure 4.5). However, at the stage of middle moulting fluid, the effect of K^+ on fat body carbonic anhydrase is reversed from that during

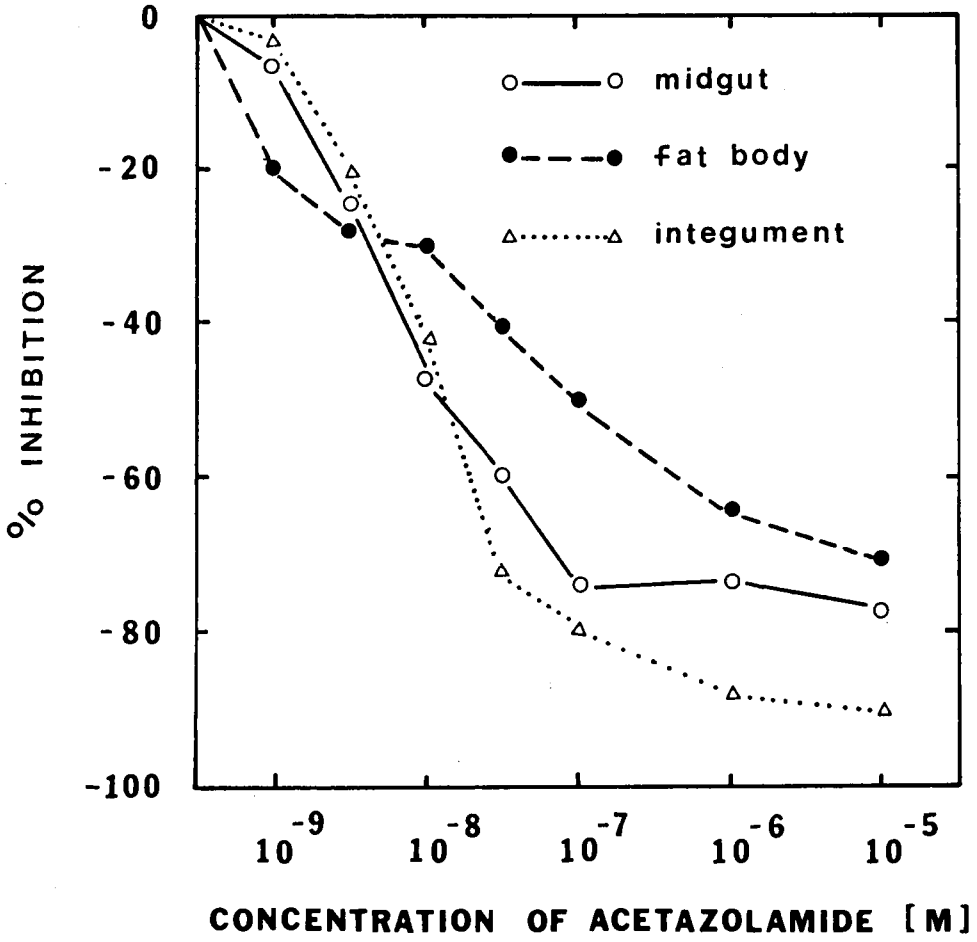


Figure 4.3. The inhibitory effects of acetazolamide on midgut, fat body and integument carbonic anhydrases from feeding fifth instar M. sexta.

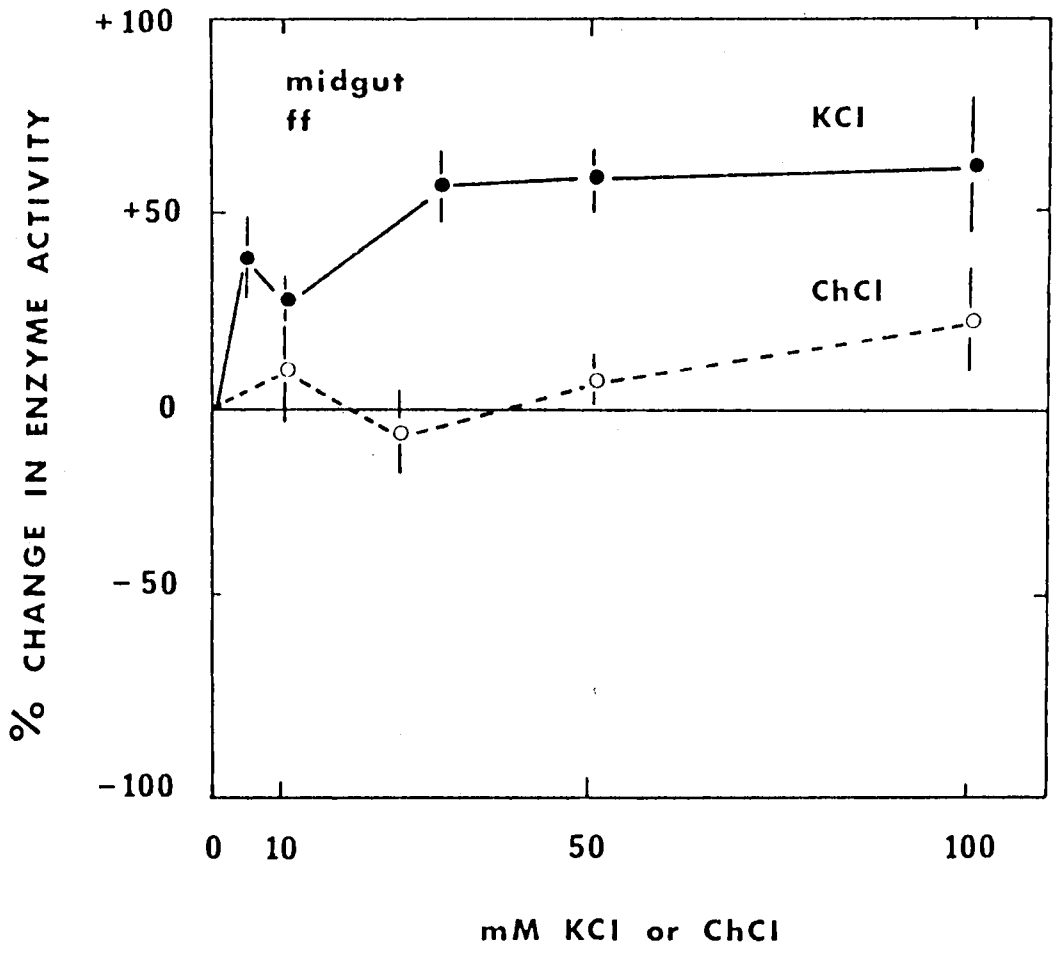


Figure 4.4. The effects of potassium chloride (KCl) and choline chloride (ChCl) on feeding fifth instar midgut carbonic anhydrase activity. Closed circles-solid line = KCl; Open circles-dashed line = ChCl.

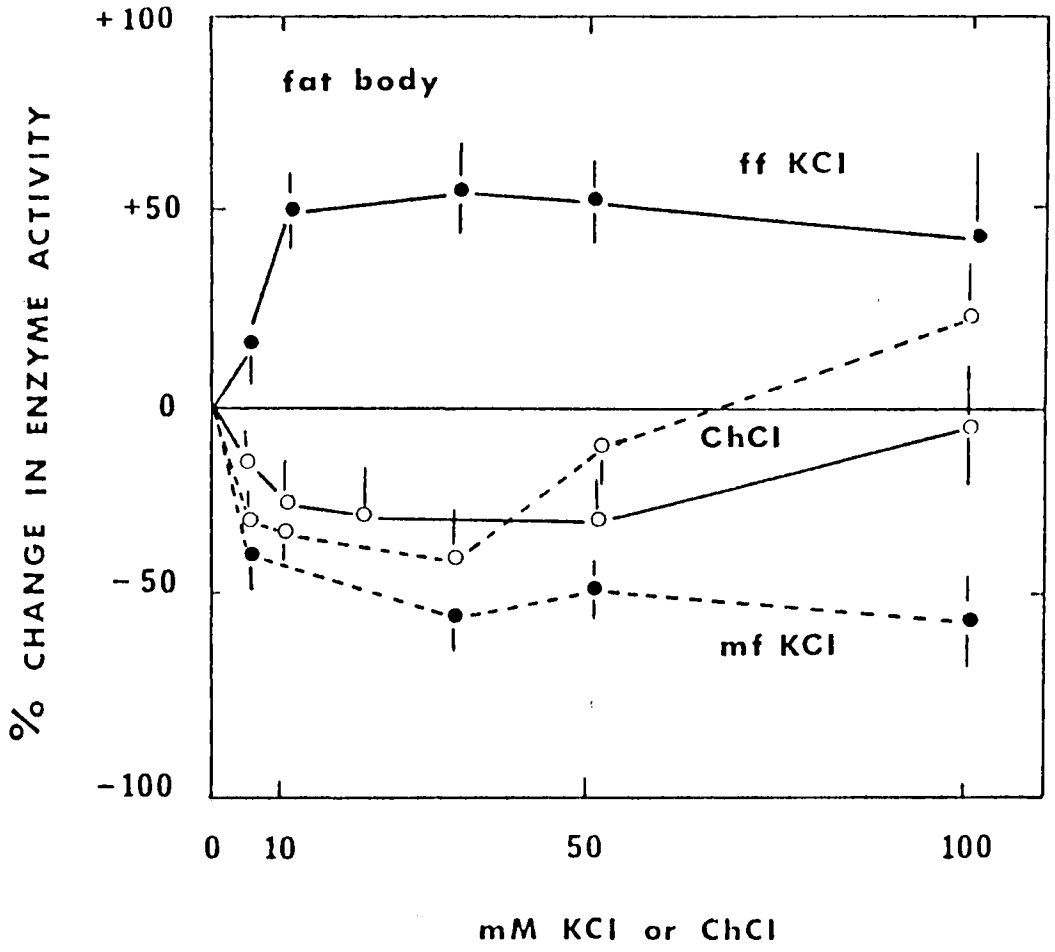


Figure 4.5. The effect of KCl and ChCl on carbonic anhydrase activity in fat body from feeding fifth (ff) instar and middle moulting fluid (mf) stages of development. Solid lines = feeding fifth instar; Dashed lines = middle moulting fluid; Closed circles = KCl; Open circles = ChCl.

the fifth instar, namely, concentrations of 10 mM or above inhibit activity by 50%.

The effects of KCl and ChCl on the integumentary enzyme are still different from patterns observed in fat body and midgut (Figure 4.6). K^+ is barely able to stimulate the integumentary enzyme during the fifth instar as it did with midgut and fat body enzymes (only a 15% activation of integumentary enzyme at 32 mM while all other concentrations have no significant effect versus 50 to 60% activation for both fat body and midgut). During the middle moulting fluid stage, integumentary enzyme acts like that of fat body in that K^+ becomes inhibitory (nearly - 30% at KCl concentrations of 32 mM or above), while ChCl effects do not differ from those found during the feeding fifth stage. The difference between fat body and integument lies in the magnitude of the effects, fat body being either stimulated or inhibited to a much greater extent than integument (contrast Figures 4.5 and 4.6).

IV. DISCUSSION

Carbonic Anhydrase in Insects

In agreement with previously reported studies (see, for instance, Florkin, 1935; Levenbook and Clark, 1950) I found no carbonic anhydrase activity in either hemolymph or muscle. The enzyme has, however, been found in midgut of several other species (gut tissue of adult orhtoptera, Anderson and March, 1956; gut tissue of Philosamia cynthia, Maren, 1967; midgut tissue of several lepidopterans, Turbeck and Foder, 1970).

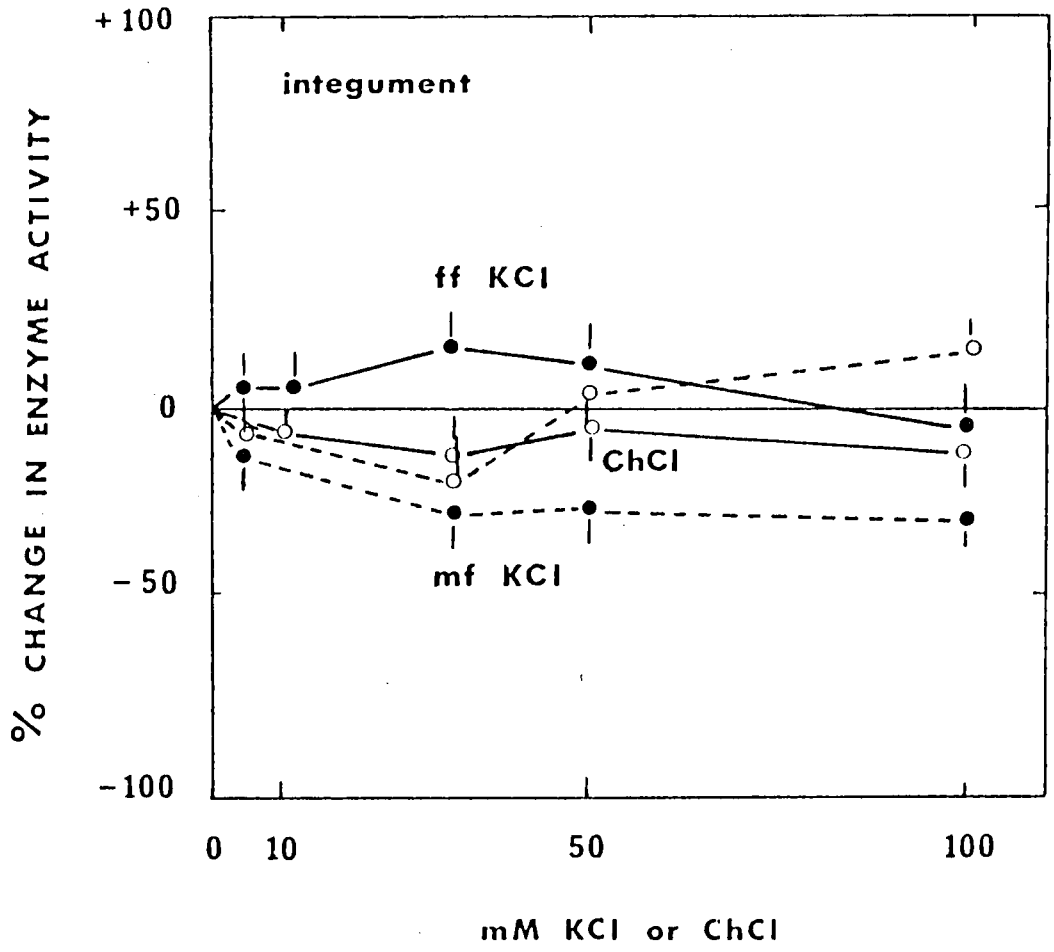


Figure 4.6. The effect of KCl and ChCl on carbonic anhydrase activity in integument from feeding fifth (ff) instar and middle moulting fluid (mf) stages of development. Solid lines = feeding fifth instar; Dashed lines = middle moulting fluid; Closed circles = KCl; Open circles = ChCl.

Unlike mammalian (see review by Bundy, 1977) and teleost (Haswell, 1967; Houston and McCarty, 1978) systems, the absence of carbonic anhydrase from blood (hemolymph) of M. sexta precludes the contamination from other tissues. Although the synthetic diet contributes no carbonic anhydrase activity, contamination from leafy remains in the gut of foliage reared animals can produce major discrepancies in the levels of midgut carbonic anhydrase actually present (see Chapter 5), since plants contain considerable quantities of this enzyme (see Bundy, 1977, for a review). Contamination from dietary sources and the fact that most studies list the levels of carbonic anhydrase as units per milligram of protein make direct comparison of enzyme levels between species impossible. I have listed my values as units per gram wet weight (the weight has been corrected for adhereing hemolymph, see Table 4.1) and as total units per animal (see Figures 4.1, p. 76, and 4.2, p. 79). I feel that this method is much more meaningful because total protein levels fluctuate drastically (see Figure 4.7). Since overall changes in total protein synthesis may or may not reflect overall changes in carbonic anhydrase levels, it seems more reasonable to list the enzyme levels as either total enzyme per animal (per tissue) or as enzyme per gram tissue weight.

My finding that the enzyme is located solely in the cytoplasm is in agreement with previously reported studies (see Berstein, et al., 1968; Karler and Woodbury, 1968; and Gay and Mueller, 1973). Not all carbonic anhydrases are localized in the cytoplasm, however. There is evidence that there are two carbonic anhydrases (cytoplasmic vs. membrane bound;

TABLE 4.1. Carbonic anhydrase units per gram wet-weight of tissue. All tissue weights have been corrected for adhereing hemolymph.

Tissue	Feeding Fifth	Pink Stripe	+ 1 Day	+ 2 Days	+ 3 Days	Early	Middle	Late	Pupae
Midgut	1.58±.062 n=7	1.74±.136 n=4	0±0 n=5	0±0 n=5	1.37±.125 n=3	1.00±.037 n=4	1.31±.021 n=10	2.01±.196 n=5	0±0 n=8
Fat Body	4.54±.273 n=7	2.91±.186 n=6	2.88±.241 n=5	2.44±.236 n=5	1.93±.198 n=4	1.75±.186 n=3	1.80±.064 n=10	1.30±.117 n=5	2.08±.091 n=8
Integument	3.45±.297 n=7	4.11±.316 n=5	3.87±.365 n=5	1.97±.184 n=5	2.07±.196 n=4	2.08±.214 n=3	3.69±.041 n=10	3.59±.170 n=5	3.93±.143 n=8

± = the standard error of the mean.

n = the number of individual determinations.

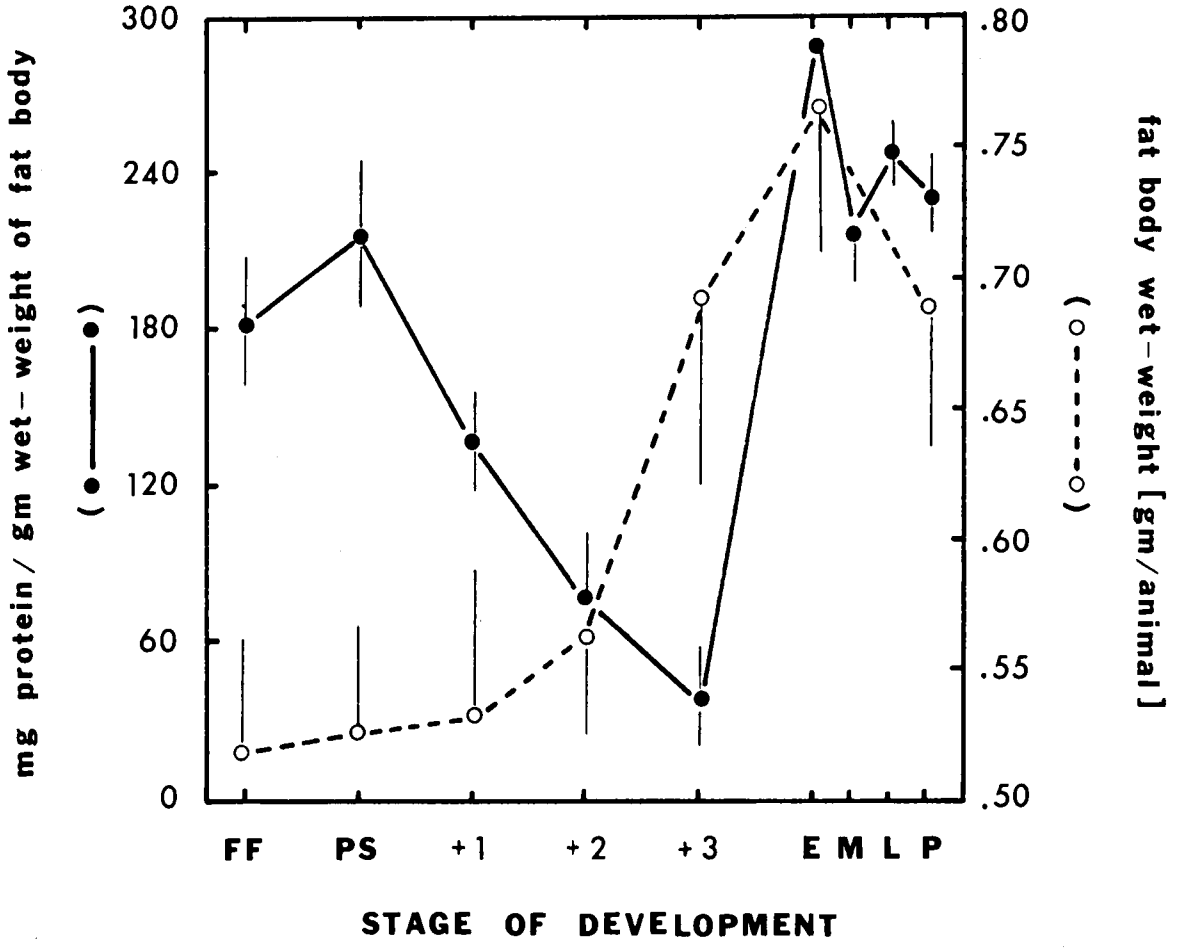


Figure 4.7. Changes in fat body wet-weight and protein content during the larval-pupal transformation of M. sexta.

see Bundy, 1977) and that the two are functionally different. I have answered this question by the use of different procedures to rupture the cells. The basic sonification procedure (see Methods) did not lyse membrane bound vesicles (based on microscopic analysis as well as data from Turbeck and Foder, 1970). When these vesicles were subsequently lysed (under stronger sonification procedures) no carbonic anhydrase activity was found. In M. sexta all carbonic anhydrase activity is confined to the cytoplasm in all tissues (see, however, Chapter 5 regarding this problem in Hyalophora cecropia).

Carbonic Anhydrase in Midgut

Midgut carbonic anhydrase levels are highest at periods when the animal is actively feeding (see Figure 4.1, p. 76). When feeding has ceased (between feeding fifth and pink stripe) the midgut carbonic anhydrase activity is correspondingly low. It is believed that HCO_3^- produced by the midgut enzyme accompanies actively transported K^+ across the gut epithelium (see Blankenmeyer, 1979). During periods of feeding, the enzyme is associated with maintenance of the ionic transport associated with the gut epithelium. Once feeding has stopped, and the gut is purged of its contents, the enzyme declines in activity. I associate the reappearance of the enzyme (just before moulting fluid production) with the movement of fluid and ions into the gut in association with the resorption of moulting fluid (see Figure 4.1) back into hemolymph. It is as if the gut is briefly "turned back on" to accommodate the influx of material (cuticular hydrolysate, moulting fluid, and so forth) coming into hemolymph (with some products

eventually moving into the gut) from the exuvial space just prior to pupation.

Carbonic Anhydrase in Fat Body

Carbonic anhydrase activity declines in fat body between feeding fifth instar and pink stripe stages (see Figure 4.1, p. 76). Thereafter, activity associated with the fat body remains relatively constant through the remainder of the larval-pupal transformation. The role of fat body carbonic anhydrase is not known, although it may be related to the presence of a glyoxylate pathway in this tissue. Bicarbonate produced in the fat body probably moves into the hemolymph where it functions as one component in the buffering system.

Carbonic Anhydrase in Integument

Carbonic anhydrase activity in the integument falls drastically between pink stripe and pink stripe + 3 days (see Figure 4.1). At the beginning of the elaboration and secretion of moulting fluid (between pink stripe + 3 days and early moulting fluid) this trend is reversed, with the activity increasing dramatically. This increase is associated with the production and secretion of moulting fluid by the integumentary epithelium. I have shown that HCO_3^- produced in the integument is actively transported to the exuvial space (i.e., away from hemolymph toward moulting fluid). The HCO_3^- so moved (1) accounts for the high levels of HCO_3^- in moulting fluid (100 mM compared to 6 mM in hemolymph, see Chapter 2); (2) acts to buffer the moulting fluid at a favorable pH (around 7.2) for hydrolytic enzymes associated with digestion of the

larval cuticle; and (3) helps provide electroneutrality for the accompanying K^+ (Chapter 7), which is also moved actively from hemolymph to moulting fluid (i.e., the same direction as net HCO_3^- movement).

Effects of Acetazolamide

In terms of acetazolamide inhibition of carbonic anhydrase in M. sexta, there appear to be at least two different responses: one associated with fat body and one associated with midgut and integument. Fat body enzyme is about 10 times less sensitive than either midgut of integument (I_{50} of fat body is 10^{-7} M versus I_{50} of midgut and integument of 10^{-8} M). These values compare favorably with I_{50}'s for bovine carbonic anhydrase which I confirmed using my assay procedures (data not shown, see Wistrand and Rao, 1968).

This information is significant in two regards. First, it shows for the first time that acetazolamide is indeed a specific inhibitor of carbonic anhydrase in insect tissues other than midgut. This report proves acetazolamide to be an adequate specific inhibitor of insect carbonic anhydrase in fat body and integument as well as midgut. Changes (usually in transport rates) seen when acetazolamide is used must represent actual inhibition of carbonic anhydrase. The difficulty lay in the observations of Kitahara, et al., (1967) that acetazolamide will inhibit Cl^- transport in the absence of carbonic anhydrase. Studies in which acetazolamide was added to bathing solutions in epithelial transport experiments (Haskell, et al., 1965; Spring, et al., 1978) assumed that carbonic anhydrase was involved. Those assumptions have now been proven correct. Secondly, the different I_{50}'s point to at

two different carbonic anhydrases in M. sexta. The fact that more than one carbonic anhydrase is present in a species is not surprising since there are at least three different human and bovine carbonic anhydrases in red blood cells alone.

Effects of K^+ and Cl^-

It has been reported that halide aniona (Cl^-, Br^-, and I^-) will activate insect midgut carbonic anhydrase (Turbeck and Foder, 1970), whereas these anions inhibit mammalian carbonic anhydrases (Koenig and Brown, 1976; Maren, et al., 1976; and Pocker and Tanaka, 1978). This study shows that it is not the anion (in this case Cl^-) which activates the insect enzyme, but rather the accompanying cation. I have also used other alkali metal cations (Cs^+, Rb^+, Li^+, and Na^+; Figure 4.8) and shown that all will activate the enzyme relative to choline chloride. It has previously been shown (Johnston and Jungreis, 1979) that the cation as well as the anion will inhibit mammalian carbonic anhydrases. It is therefore imperative that proper controls be employed in conducting experiments dealing with the effects of chloride salts on carbonic anhydrase activity. In this regard we have employed choline as a substitute for K^+, because it is calculated to be of such large molecular size as to be excluded from all catalytic and allosteric site interaction. By using choline chloride the effect of KCl on enzyme activity can therefore be separated into a K^+ and a Cl^- effect.

In the case of midgut, K^+ activates the enzyme by ca. 50% at concentrations above 30 mM (see Figure 4.3, p. 81), while choline chloride has no significant effect ($p \leq .005$ at all concentrations below

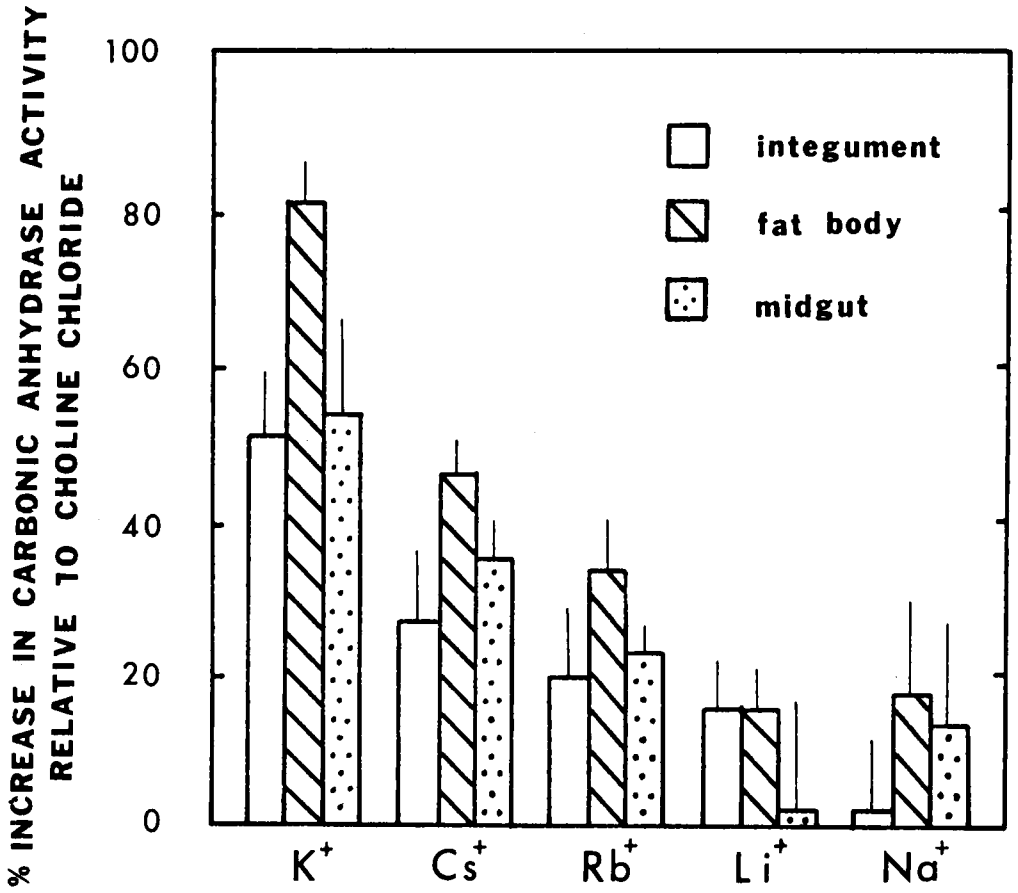


Figure 4.8. Effects of various alkali metal cations on carbonic anhydrase activity in integument, fat body and midgut from feeding fifth instar M. sexta. Values are listed as percentage increase in activity relative to choline chloride. All chloride salts were present at 32 mM. The CO_2 concentration was 30.6 mM. Each large bar is the mean of 4 separate determinations; each narrow line is the standard error of the mean.

about 100 mM). Since midgut carbonic anhydrase is present in such small quantities at moulting fluid stages (see Figure 4.1, p. 76, and 4.2, p. 79) we were unable to obtain sufficient enzyme from one animal to test the effects of K^+ and Cl^- at these stages. However, we have compared the effects of K^+ and Cl^- on both fat body and integument carbonic anhydrases at both feeding fifth and moulting fluid stages.

Both fat body and integumentary carbonic anhydrase undergo a change between feeding fifth instar and moulting fluid, so that K^+ becomes an inhibitor rather than an activator of the enzymes at moulting fluid stages. At the feeding fifth stage, fat body carbonic anhydrase is activated by about 50% at KCl concentrations above 10 mM (physiological concentrations of K^+ in M. sexta hemolymph is about 30 mM while Cl^- is around 25 mM). At middle moulting fluid stages, the fat body enzyme is inhibited by 50% at these same KCl concentrations (physiological concentrations of K^+ and Cl^- in hemolymph remain unchanged between feeding fifth instar and middle moulting fluid stages). This is clearly a K^+ effect by inference from the response of the enzyme to choline chloride (Figure 4.5, p. 83).

The same trend is seen with integumentary carbonic anhydrase, although to a much smaller degree. The enzyme is activated by KCl (about 20% at 30 mM, see Figure 4.6, p. 85) during the feeding fifth stage, but inhibited (about 40%) by the same concentration during the middle moulting fluid stage. Since chloride is clearly not the cause of this stage specific response (Figure 4.6), K^+ must be the causative agent.

The cause of this differential response is not known, but could be related to changing hormone titers as the animal undergoes pupation. Such a hormonal effect could work via a stage specific protein kinase (such a kinase has been implicated in controlling the enzymatic activity of carbonic anhydrase in other species; see Bundy, 1977) which would modify the production of carbonic anhydrase in such a manner that allosteric interaction with K^+ becomes reversed. This reversal could serve as a regulator in the HCO_3^- - K^+ transport system across the integumentary epithelium during periods of moulting fluid secretion and resorption.

It therefore appears that at least two different carbonic anhydrases are produced: one in fat body and one in integument. The first type (K^+ activated) is produced during the last larval instar (based on acetazolamide effects, the K^+ activated enzyme in fat body is different from that produced by the integument), while the second type (K^+ inhibitory) is produced during the time of moulting fluid secretion and resorption.

In summary, carbonic anhydrase was detected only in midgut, fat body and integument of the tobacco hornworm during the larval-pupal transformation. During the period of the last larval instar each tissue appears to produce a specific enzyme (i.e., each is different from the other). All three are activated by physiological levels of K^+ and either inhibited, or unaffected by physiological levels of Cl^-. During the latter stages of the larval-pupal transformation a second type of carbonic anhydrase is produced by the fat body. At these

stages the integument also produces a second type of enzyme (different from the second type of enzyme produced by the fat body). Both of these second enzyme types are unique in that K^+ no longer activates them, but rather inhibits.

CHAPTER 5

CARBONIC ANHYDRASE ACTIVITY DURING THE LARVAL-PUPAL TRANSFORMATION OF BOTH FOLIAGE AND LAB REARED *Hyalophora cecropia*: EFFECTS OF K^+ AND Cl^- ON MIDGUT, FAT BODY AND INTEGUMENTARY ENZYMES

I. INTRODUCTION

Bicarbonate has been implicated in the production and movement of fluids in several insect systems. The labial gland of *Antheraea pernyi* has been shown to secrete a potassium bicarbonate salt solution (Kafatos, 1968). The alkaline fluid expectorated by tobacco hornworms, *Manduca sexta*, during formation of the pupation chamber probably involves H^+/HCO_3^- transport by the salivary gland (= silk gland) or gut (Lockstein, et al., 1978). Other studies on fluid and ion movement by the gut of *Hyalophora cecropia* during the larval stages have also implicated HCO_3^- (Haskell, Clemons and Harvey, 1965; Harvey, Haskell and Nedergaard, 1968). In addition, HCO_3^- may play a major role in the control of fluid and ion movement across the locust, *Schistocerca gregaria*, rectum (Williams, et al., 1978; Goh and Phillips, 1977).

Bicarbonate not only plays a role in fluid movement in the digestive and excretory systems, but also in control of moulting fluid elaboration. Jungreis and Harvey (1975) proposed that HCO_3^- is the counter ion passively transported with actively transported K^+ during

the secretion of moulting fluid by the larval-pupal integument of H. cecropia. I have found in M. sexta (Chapter 7) that HCO_3^- is non-electrogenically coupled to K^+ and H^+ ion movement during moulting fluid secretion and that the active transport of HCO_3^- may drive the resorption of moulting fluid.

In a recent study of some of the anionic components in hemolymph and moulting fluid in the silkmoth, Hyalophora cecropia (Chapter 3), it was noted that bicarbonate levels in hemolymph were at 35 mM, while those in moulting fluid were over 100 mM. The origin of the hemolymph bicarbonate is undertain, since hemolymph is reported to be without carbonic anhydrase activity (Turbeck and Foder, 1970). The origin of moulting fluid bicarbonate is also a mystery, since carbonic anhydrase activity has never been measured in either moulting fluid, integument or fat body.

In this report carbonic anhydrase activity in midgut, fat body and integument was determined at a variety of developmental stages during the larval-pupal transformation of the silkworm, Hyalophora cecropia. After determining total and tissue specific enzymatic activities, the effects of K^+ and Cl^- on the enzymes were investigated in an effort to gain insight into some of the factors which regulate that activity. Since bicarbonate plays an important role in many physiological systems in the insect, our understanding of the sites of its production and the modes of its regulation will surely enhance our knowledge of these systems.

II. MATERIALS AND METHODS

Lab Reared Silkmoths

Silkmoths were reared in the laboratory on the artificial diet of Riddiford (1968) at 25°C on a light regimen of 12L:12D. Animals were staged and hemolymph and moulting fluid were collected by the methods of Jungreis (staging, 1979; fluid collection, 1974).

Foliage Reared Silkmoths

Silkmoths were reared on wild cherry leaves (Purnus serotina) in both the laboratory and outdoors according to the procedure outlined in Chapter 3.

Carbonic Anhydrase Activity

Total carbonic anhydrase activity was determined in midgut, fat body, integument, muscle, silk gland, larval cuticle, pupal cuticle, gut contents, hemolymph and moulting fluid from each animal at the appropriate stages in development. Carbonic anhydrase was assayed according to Wilbur and Anderson (1948) as modified by Johnston and Jungreis (1979, 1980). The final CO_2 concentration in the assay mixture was either 30.6 mM (tissue assays) or 15.3 mM (alkali metal cation effects, see below).

Effects of K^+ and Cl^-

The effects of potassium chloride (KCl) and choline chloride (ChCl) on carbonic anhydrase activity were determined on midgut, fat body and integumentary at the feeding fifth and instar and middle moulting fluid

stages in both foliage and lab reared silkmoths. The assays were carried out as previously described with the appropriate concentration of salt added to the final incubation mixture.

Since choline is calculated to be of such large molecular size as to be excluded from all allosteric site interaction, its substitution for K^+ allows for the separation of chloride effects from K^+ effects (see Johnston and Jungreis, 1979 and 1980).

Effects of Acetazolamide

Acetazolamide, a known specific inhibitor of carbonic anhydrase (Chapter 5; Maren, 1971) was added to the final incubation mixture at concentrations ranging from 10^{-10} to 10^{-5} M. The effects of this specific inhibitor were determined on both foliage and lab reared midgut, fat body and integumentary enzymes during the feeding fifth stage. The effects of acetazolamide, in the same concentration range, were also tested on gut contents from feeding fifth instar animals under both rearing conditions.

Effects of Li^+, Na^+, Cs^+ and Rb^+

A variety of alkali metal cations (Li^+, Na^+, Cs^+ and Rb^+) were used to determine the specificty of cation effects on midgut, fat body and integumentary enzymes in foliage reared H. cecropia at both feeding fifth and moulting fluid stages. All cations were added as their chloride salts at a final concentration of 32 mM. The final CO_2 concentration in the assay mixture was reduced from 30.6 mM (the CO_2 concentration used for tissue assays) to 15.3 mM in order to allow for

maximal effect of the cation, while simultaneously maintaining the CO_2 levels within the physiological range of H. cecropia hemolymph (H. cecropia hemolymph has 10-33 mM HCO_3^- at a pH of ca. 6.7 during the larval-pupal transformation; see Johnston and Jungreis, 1980).

Dialysis of Foliage Midgut

Foliage reared H. cecropia midguts were prepared in the usual fashion (see Johnston and Jungreis, 1979, 1980) for the carbonic anhydrase assay procedure. Half of the supernatant fraction (ca. 2.5 ml with approximately 1 unit of carbonic anhydrase activity) from each midgut was used as a control (carbonic anhydrase activity tested in the absence and presence of 50 mM KCl) while the remaining half was dialyzed against 50 ml of 15 mM Hepes Buffer. The dialysis was carried out at 3°C in an Amicon filtration chamber (Amicon Model 52, 75 psi max; particles with a molecular weight greater than 10,000 are excluded by the PM 10 filter) with constant stirring. A constant filtration rate (.5 ml/minute) was maintained by pressurizing the chamber to 50 psi with nitrogen.

When the initial volume of supernatant (2.5 ml) was remaining in the filtration chamber the dialysis was stopped (room pressure restored) and the carbonic anhydrase activity of the unfiltered portion assayed in the presence and absence of 50 mM KCl. Ninety-two percent of the initial carbonic anhydrase activity remained in the unfiltered portion following filtration of 50 mls of 15 mM Hepes Buffer (pH 8.35). No carbonic anhydrase activity was present in any portion of the filtrate.

III. RESULTS

Carbonic Anhydrase Activity Throughout the Larval-Pupal Transformation

Midgut. Carbonic anhydrase activity in the midgut is highest at the feeding fifth stage in both foliage and lab reared silkmoths (3.1 units/gm wet weight or 2.1 total tissue units in foliage animals, see Tables 5.1 and 5.2; and 2.8 units/gm wet-weight or 1.9 total tissue units in lab reared animals, see Tables 5.3 and 5.4). As feeding ceases and the animal purges its gut contents prior to the initiation of spinning, the carbonic anhydrase activity in the gut falls drastically in both rearing groups (from 3.1 $\longrightarrow$ 0.7 and 2.8 $\longrightarrow$ 0.6 units/gm wet-weight in foliage + diet reared animals, see Tables 5.1 and 5.3). This decline continues until apolysis (about 3 days after the initiation of spinning) when the gut is without detectible enzyme activity (see Tables 5.1 and 5.2 for foliage; Tables 5.3 and 5.4 for lab). No carbonic anhydrase activity was found in the midgut at any stage between apolysis and the newly ecdysed pupa under either rearing condition.

Virtually 100% of the carbonic anhydrase activity in midgut was found in the cytoplasm. By using differential cell lysing procedures (see Johnston and Jungreis, 1980) we found that no subcellular vesicle contained enzyme activity. Extreme caution must be taken, however, to exclude all gut content contamination from foliage reared H. cecropia midguts (no carbonic anhydrase activity was found in gut contents of synthetic diet reared H. cecropia). We have found that the gut contents of foliage reared silkmoths contain up to 700 units of carbonic

TABLE 5.1. Units of carbonic anhydrase per gram wet-weight of tissue in foliage reared H. cecropia. All tissue weights were corrected for adhereing hemolymph.

Stage	Midgut	Fat Body	Integument
Feeding Fifth	3.06±.488 n=15	2.25±.386 n=15	12.98±.477 n=15
Day of Spin	.672±.078 n=3	4.61±.569 n=3	11.63±.986 n=3
Spin + 1 Day	.583±.065 n=4	4.31±.359 n=4	15.40±1.64 n=4
Spin + 2 Days	.188±.023 n=4	4.30±.408 n=4	17.67±1.96 n=4
Apolysis	0 n=3	7.27±.298 n=3	20.27±2.58 n=3
Apolysis + 1 Day	0 n=3	6.24±.547 n=3	24.55±3.29 n=3
Apolysis + 2 Days	0 n=3	7.43±.526 n=3	23.43±2.87 n=3
Apolysis + 3 Days	0 n=3	6.65±.764 n=3	20.71±2.67 n=3
Apolysis + 4 Days	0 n=3	6.71±.851 n=3	18.86±1.91 n=3
Early Moulting	0 n=3	2.86±.248 n=3	18.67±1.77 n=3
Middle Moulting	0 n=8	3.53±.106 n=8	18.95±1.84 n=8
Late Moulting	0 n=3	1.54±.038 n=3	15.56±2.60 n=3
Pupae	0 n=5	1.90±.099 n=5	7.60±.563 n=5

n = number of individual determinations; numbers are means ± the standard error of the mean.

TABLE 5.2. Total carbonic anhydrase units per animal per tissue throughout the larval-pupal transformation of foliage reared H. cecropia. Tissue weights were corrected for adhereing hemolymph and normalized to a feeding fifth instar body weight of 15 gm (see text). Integument weight does not include cuticle.

Stage	Midgut	Fat Body	Integument	Total
Feeding Fifth	2.10±.335 n=15	2.03±.347 n=15	19.48±.716 n=15	23.60±1.40 n=15
Day of Spin	.329±.038 n=3	5.07±.626 n=3	12.79±1.08 n=3	18.19±1.74 n=3
Spin + 1 Day	.255±.028 n=4	4.74±.395 n=4	15.09±1.80 n=4	20.09±2.22 n=4
Spin + 2 Days	.072±.009 n=4	4.73±.449 n=4	15.20±2.16 n=4	20.00±2.62 n=4
Apolysis	0 n=3	8.00±.373 n=3	15.20±1.94 n=3	23.20±2.32 n=3
Apolysis + 1 Day	0 n=3	7.80±.766 n=3	17.80±2.39 n=3	25.60±3.16 n=3
Apolysis + 2 Days	0 n=3	10.40±.815 n=3	16.40±2.01 n=3	26.80±2.83 n=3
Apolysis + 3 Days	0 n=3	10.31±1.31 n=3	14.50±1.87 n=3	24.81±2.79 n=3
Apolysis + 4 Days	0 n=3	11.41±1.45 n=3	13.20±1.34 n=3	24.61±2.79 n=3
Early Moulting	0 n=3	5.29±.457 n=3	11.20±1.06 n=3	16.50±1.52 n=3
Middle Moulting	0 n=8	6.53±.196 n=8	11.37±1.11 n=8	17.90±1.31 n=8
Late Moulting	0 n=3	2.85±.070 n=3	9.34±1.56 n=3	12.20±1.63 n=3
Pupae	0 n=5	3.80±.198 n=5	3.80±.282 n=5	7.60±.480 n=5

n = number of individual determinations; numbers are means ± standard errors of the mean.

TABLE 5.3. Units of carbonic anhydrase per gram wet weight of tissue throughout the larval-pupal transformation of lab reared H. cecropia. All tissue weights were corrected for adhereing hemolymph.

Stage	Midgut	Fat Body	Integument
Feeding Fifth	2.78±.879 n=6	3.15±.447 n=6	14.07±1.43 n=6
Day of Spin	.561±.271 n=3	1.93±.095 n=3	10.18±2.06 n=3
Spin + 1 Day	.501±.222 n=3	2.53±.342 n=3	13.27±1.68 n=3
Spin + 2 Days	.283±.186 n=3	1.54±.189 n=3	16.74±1.44 n=3
Apolysis	0 n=6	7.64±1.10 n=6	35.20±2.64 n=6
Apolysis + 1 Day	0 n=3	2.40±.323 n=3	11.86±.860 n=3
Apolysis + 2 Days	0 n=3	4.79±.765 n=3	13.57±1.95 n=3
Apolysis + 3 Days	0 n=3	4.06±.452 n=3	10.43±1.73 n=3
Apolysis + 4 Days	0 n=3	4.00±.556 n=3	10.29±1.72 n=3
Early Moulting	0 n=3	9.73±1.85 n=5	30.00±2.89 n=5
Middle Moulting	0 n=8	8.95±1.97 n=8	13.33±1.76 n=8
Late Moulting	0 n=3	8.34±1.95 n=3	10.93±.958 n=3
Pupae	0 n=3	10.10±2.03 n=3	9.60±1.43 n=3

n = the number of individual determinations; numbers are means ± the standard error of the mean.

TABLE 5.4. Total carbonic anhydrase units per animal tissue through the larval-pupal transformation in lab reared H. cecropia (see Table 5.2).

Stage	Midgut	Fat Body	Integument	Total
Feeding Fifth	1.91±.604 n=6	2.84±.402 n=6	21.10±2.15 n=6	25.85±3.16 n=6
Day of Spin	.274±.133 n=3	2.12±.105 n=3	11.20±2.27 n=3	13.60±2.51 n=3
Spin + 1 Day	.220±.097 n=3	2.78±.376 n=3	13.00±1.65 n=3	16.00±2.12 n=3
Spin + 2 Days	.109±.072 n=3	1.69±.208 n=3	14.40±1.24 n=3	16.20±1.52 n=3
Apolysis	0 n=6	8.40±1.21 n=6	26.40±1.98 n=6	34.80±3.19 n=6
Apolysis + 1 Day	0 n=3	3.00±.404 n=3	8.59±.624 n=3	11.60±1.03 n=3
Apolysis + 2 Days	0 n=3	6.71±1.07 n=3	9.50±1.34 n=3	16.21±2.41 n=3
Apolysis + 3 Days	0 n=3	6.29±.701 n=3	7.30±1.21 n=3	13.60±1.91 n=3
Apolysis + 4 Days	0 n=3	6.80±.945 n=3	7.20±1.20 n=3	14.00±2.15 n=3
Early Moulting	0 n=3	18.00±3.42 n=3	18.00±1.73 n=3	36.00±5.15 n=3
Middle Moulting	0 n=8	16.56±3.64 n=8	8.00±1.06 n=8	24.56±4.70 n=8
Late Moulting	0 n=3	15.43±3.60 n=3	6.56±.575 n=3	21.99±4.18 n=3
Pupae	0 n=3	20.20±4.06 n=3	4.80±.715 n=3	25.00±4.78 n=3

n = number of individual determinations; numbers are means ± standard errors of the mean.

anhydrase activity per gram wet-weight (range 400 to 700 units/gm wet-weight). This value reflects the enzymatic activity found in the leaves of the wild cherry tree (Prunus serotina). We have also found that the plant enzyme appears to be associated not only with the cytoplasm, but also with some subcellular vesicle as well. It is probable that recent studies of lepidopteran midgut carbonic anhydrase (Turbeck and Foder, 1970), in which a non-cytoplasmic enzyme was found at levels 10 times higher than we determined, are, in reality, studies on plant carbonic anhydrases.

Fat body. Fat body carbonic anhydrase activity increases in a step-like fashion in foliage reared H. cecropia. Significant increases ($p < 0.05$) in total tissue activity occur primarily between (1) feeding fifth and day of spin (from 2 to 5 units); (2) spin + 2 days and apolysis (from 4.7 to 8 units); and (3) apolysis + 1 day and apolysis + 2 days (7.8 to 10.4 units, see Table 5.2). Between apolysis + 2 days and apolysis + 4 days the enzyme activity in foliage reared animals is relatively constant. Between apolysis + 4 days and the early moulting fluid stage there is a significant decrease in total carbonic anhydrase activity (11.4 units at apolysis + 4 days to 5.3 units at early moulting fluid, $p < 0.05$, see Table 5.2). This decrease continues throughout the moulting fluid process and into the pupal stage (see Table 5.2).

Carbonic anhydrase activity in lab reared silkmoths is very different from their foliage reared counterparts. Between feeding fifth instar and spin + 2 days the enzyme activity does not increase as in

foliage reared animals (compare Table 5.2 with 5.4). Between spin + 2 days and apolysis, however, there is a significant increase in fat body carbonic anhydrase activity (1.7 total tissue units at spin + 2 days to 8.4 total units at apolysis, $p < 0.05$, see Table 5.4). This increased activity is transient in nature, so that within 24 hours (i.e., by apolysis + 1 day) the levels of carbonic anhydrase have dropped to around 3 units (see Table 5.4). Like similarly staged foliage silkmoths, lab reared animals have relatively constant levels of carbonic anhydrase activity in fat body between apolysis + 2 days and apolysis + 4 days (although lab animals have significantly lower levels than do foliage animals at these stages, compare Tables 5.2 and 5.4).

From apolysis + 4 days until pupation the fat body levels of carbonic anhydrase in lab reared H. cecropia are very different from similarly staged foliage animals. Between apolysis + 4 days and early moulting fluid (a period of approximately 24 hours) total fat body carbonic anhydrase activity triples in lab reared animals (during this same developmental period in foliage reared animals fat body levels decline by 50%, compare Tables 5.2 and 5.4). Between early and late moulting fluid stages (a period of approximately 24 to 36 hours) fat body carbonic anhydrase activity declines slightly (18 units vs. 15 units, see Table 5.4). As the animal completes the resorption of moulting fluid and sheds the larval cuticle (the larval pupal ecdysis) the levels of carbonic anhydrase activity in fat body increase to over 20 units. In contrast to this response in lab reared animals, foliage reared silkmoths have significantly decreasing levels of carbonic

anhydrase in their fat body during moulting fluid elaboration and in new pupae.

Integument. Total integumentary carbonic anhydrase in foliaged reared H. cecropia declines sharply between the fifth instar and initiation of spinning (Figure 5.1; Table 5.2, p. 104). Following initiation of spinning but before completion of the outer cocoon (a span of about 12 hours) the carbonic anhydrase activity in integument increases slightly (12.8 to 15 units, see Table 5.2 and Figure 5.1) and then remains constant for the next two days (spin + 1 day to apolysis). A second period of increased activity (the first having come between spin and spin + 1 day) occurs between apolysis and apolysis + 1 day with levels as high as 18 units being reached (Table 5.2 and Figure 5.1). Thereafter, from apolysis + 1 day until early moulting fluid (a span of 4 days), the levels of carbonic anhydrase in integument of foliage reared silkmoths decline at a constant rate (a daily loss of about 10% of the total activity present at apolysis + 1 day, see Table 5.2 or Figure 5.1). This loss in activity slows for about 8 to 12 hours between early and middle moulting fluid (a time when the rate of moulting fluid secretion is maximal) and then continues at a greater rate until the larval-pupal ecdysis is complete (11.2 units at early moulting fluid, 11.4 units at middle moulting fluid, but only 3.8 units in the newly ecdysed pupa, see Table 5.2).

Total carbonic anhydrase present in integument for the first three days of the larval pupal transformation is the same for artificial diet versus foliage reared silkmoths. Thereafter, as a function of diet,

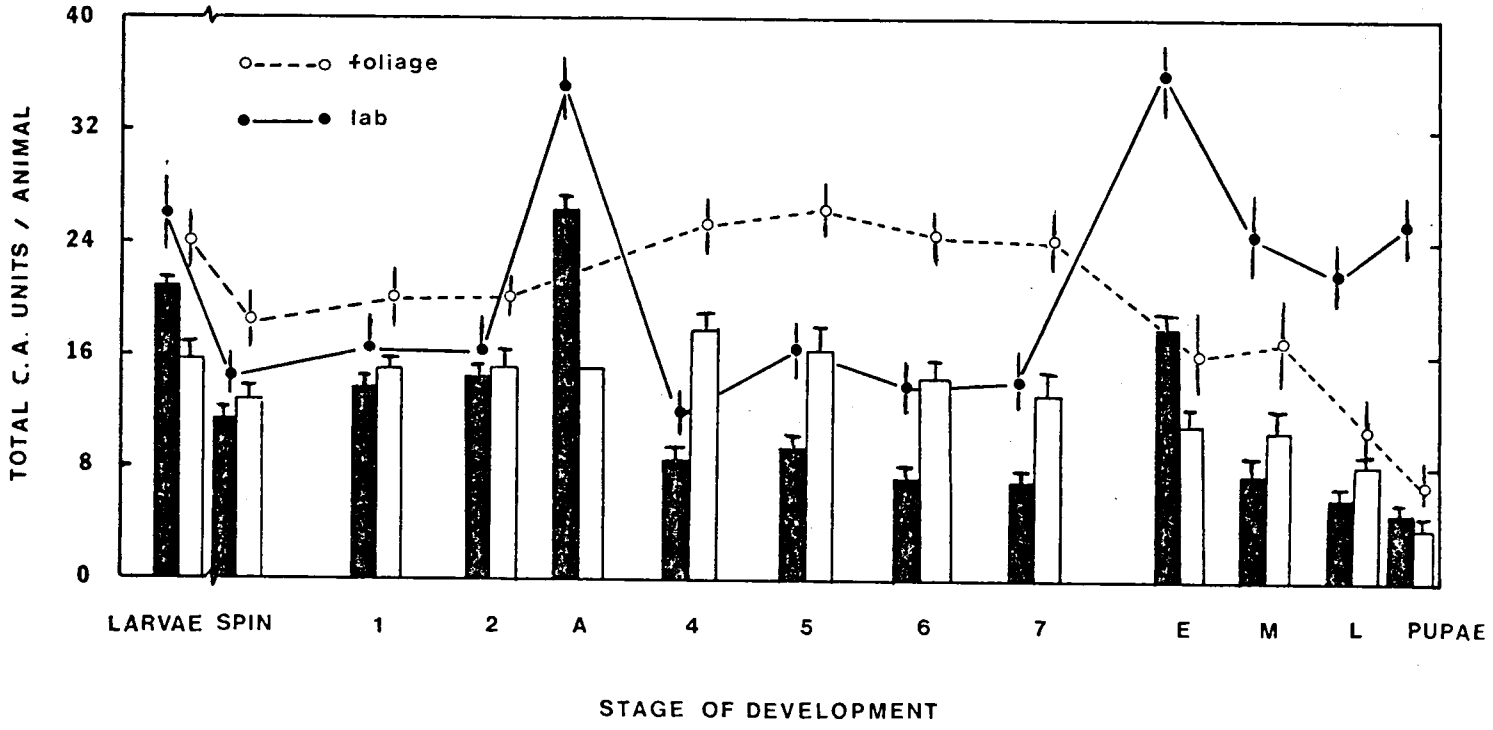


Figure 5.1. Total carbonic anhydrase units per animal and per integument throughout the larval-pupal transformation of H. cecropia. Closed circles-solid lines = total enzyme units in synthetic diet reared animals; open circles-dashed lines = total enzyme units in foliage reared animals; solid bars represent the number of enzyme units present in the integument of synthetic diet animals; open bars represent the number of enzyme units present in the integument of foliage animals.

significant differences are noted at every successive stage in development up to but not including the newly ecdysed pupa (Figure 5.1). Integument from artificial diet as well as foliage reared silkworms experiences a sharp decline in the carbonic anhydrase activity between the fifth instar and the initiation of spinning (21 units vs. 11 units, see Table 5.4). Between the day of spinning and spinning + 2 days, levels of carbonic anhydrase change little, although foliage reared insects have 25% more activity (11.2 vs. 14.4 units, respectively, Tables 5.2 and 5.4). The onset of apolysis triggers changes in total activity that are very much diet specific (Figure 5.1). A doubling of the activity of integument in synthetic diet reared insects only occurs on the of apolysis (Table 5.4 and Figure 5.1), followed 24 hours later by a 60% loss of activity such that foliage reared insects now have twice the total and twice the integumentary activity of diet reared insects (Figure 5.1 and Tables 5.2, 5.4). Activity in silkworms from both diets then remains constant through apolysis + 4 days, when integument from lab but not foliage reared insects exhibits a 150% increase (14 to 36 units) in activity (Figure 5.1 and Table 5.4), which declines as dramatically as it rose until only 5 units remain in newly ecdysed pupae. Apart from the tremendous increase in activity noted in early moulting animals, foliage reared insects also experience a dramatic 70% loss of integumentary carbonic anhydrase between the day of apolysis + days and newly ecdysed pupa (Table 5.2 and Figure 5.1).

Effects of Acetazolamide

In midgut, acetazolamide is more effective in inhibiting carbonic anhydrase from foliage than from diet insects (I_{50}'s of 10^{-8} M versus 10^{-7} M, respectively; Figure 5.2). Acetazolamide does not inhibit carbonic anhydrase present as gut contents in foliage reared animals, and in fact the activity in wild cherry leaves is actually elevated 20% in the presence of 10^{-8} M acetazolamide. This observation can be extremely useful in differentiating between nascent midgut enzyme and contaminating plant enzyme. Midgut carbonic anhycrase is not alone in exhibiting a diet specific I_{50} towards acetazolamide, since I_{50}'s for foliage versus diet reared fat body enzymes are 10^{-7} M and 10^{-8}, respectively (see Figure 5.2). Similarity of I_{50}'s in foliage versus diet reared insects should not be interpreted as evidence that the same enzyme is present in both tissues, as will be documented below. Integumentary carbonic anhydrases have the same I_{50}'s regardless of diet (3×10^{-8} M) which is intermediate in value between the midgut and the fat body enzymes.

Effects of K^+ and Cl^-

Midgut. The addition of KCl at concentrations between 5 to 50 mM (CO_2 levels of 30.6 mM) to midgut carbonic anhydrase in vitro causes a near linear increase in activation of the enzyme, an effect seen only in foliage reared H. cecropia (Figure 5.3). The midgut enzyme is inhibited by almost 50% at KCl concentrations below 50 mM in diet reared animals (Figure 5.3). This KCl activation in foliage reared animals is a

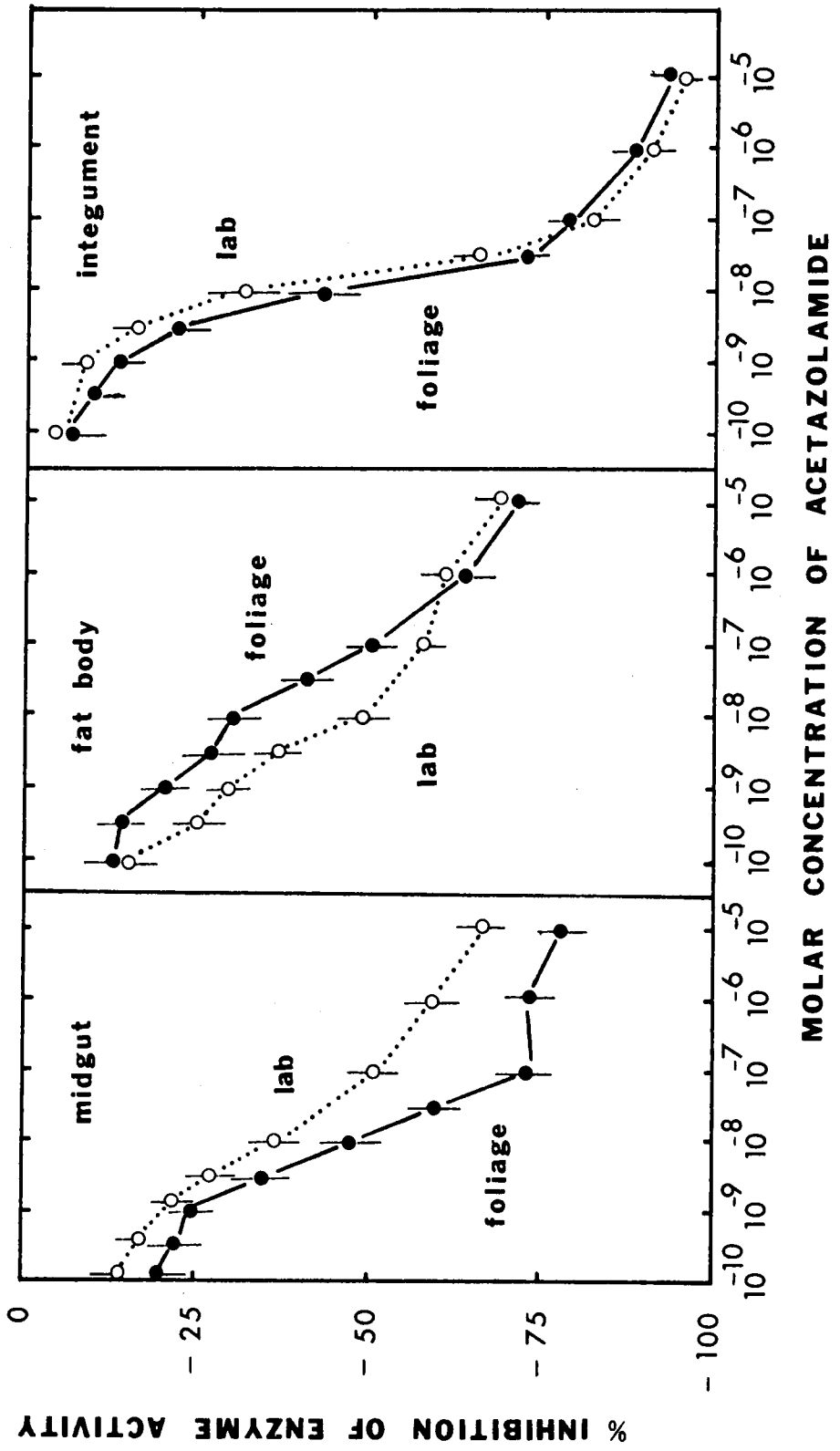


Figure 5.2. Inhibitory effect of acetazolamide on carbonic anhydrase activity from midgut, fat body and integument from both synthetic diet and foliage reared H. cecropia.

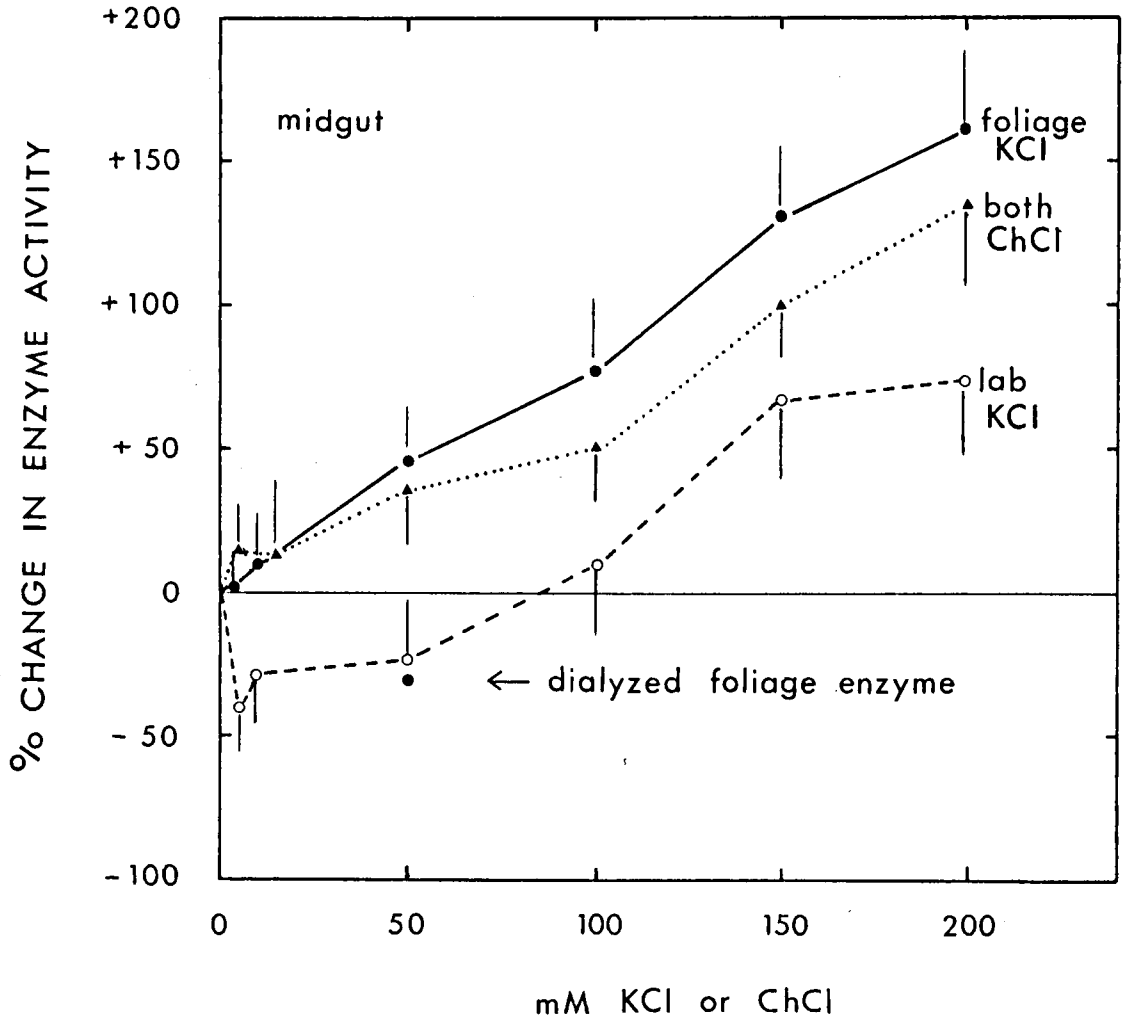


Figure 5.3. Effect of potassium chloride (KCl) and choline chloride (ChCl) on fifth instar midgut carbonic anhydrase activity from both synthetic diet and foliage reared H. cecropia. The line marked both ChCl indicates that the effect of ChCl on foliage enzyme is not significantly different from lab enzyme. The point labeled dialyzed foliage enzyme represents the effect of 50 mM KCl on foliage midgut carbonic anhydrase following dialysis (see Methods section).

chloride effect (70 to 80% of the activation), while the inhibition exhibited by diet reared midgut is caused by K^+ (see the line for choline chloride (ChCl) in Figure 5.3).

Midgut from foliage reared silkworms was dialyzed against 15 mM Hepes Buffer through a semipermeable membrane (see Methods) and became transformed into "diet type" carbonic anhydrase, in that the enzyme previously activated (50%) by 50 mM KCl was now inhibited (40%) by 50 mM KCl (Figure 5.3). When lab reared midgut was sonicated in the 15 mM Hepes filtrate from the foliage midgut dialysis experiment, KCl activated the enzyme by 30% (in contrast to the normal 40% inhibition). Thus, foliage midgut carbonic anhydrase from synthetic diet carbonic anhydrase by the presence of a dialysable non-labile component, whose addition causes a transformation of the lab enzyme to the foliage enzyme.

Fat body. The effects of $K^+(Cl^-)$ and $(Ch^+)Cl^-$ on the fifth instar fat body carbonic anhydrase in both foliage and lab reared H. cecropia are shown in Figure 5.4. Enzymes from both foliage and diet reared tissues are inhibited 40% at KCl concentrations between 10 and 100 mM. In foliage animals only, this inhibition is due to the additive effects of K^+ and Cl^- (see the line marked foliage ChCl, Figure 5.4), while in diet reared animals, since Cl^- greatly stimulates (80%) fat body carbonic anhydrase, inhibition seen in the presence of KCl is due entirely to K^+ (see the line marked lab ChCl, Figure 5.4).

A comparable study was done with fat body carbonic anhydrase from foliage animals at the middle moulting fluid stage (Figure 5.5). KCl inhibits 20 to 40% at concentrations between 10 and 200 mM, with 100 mM

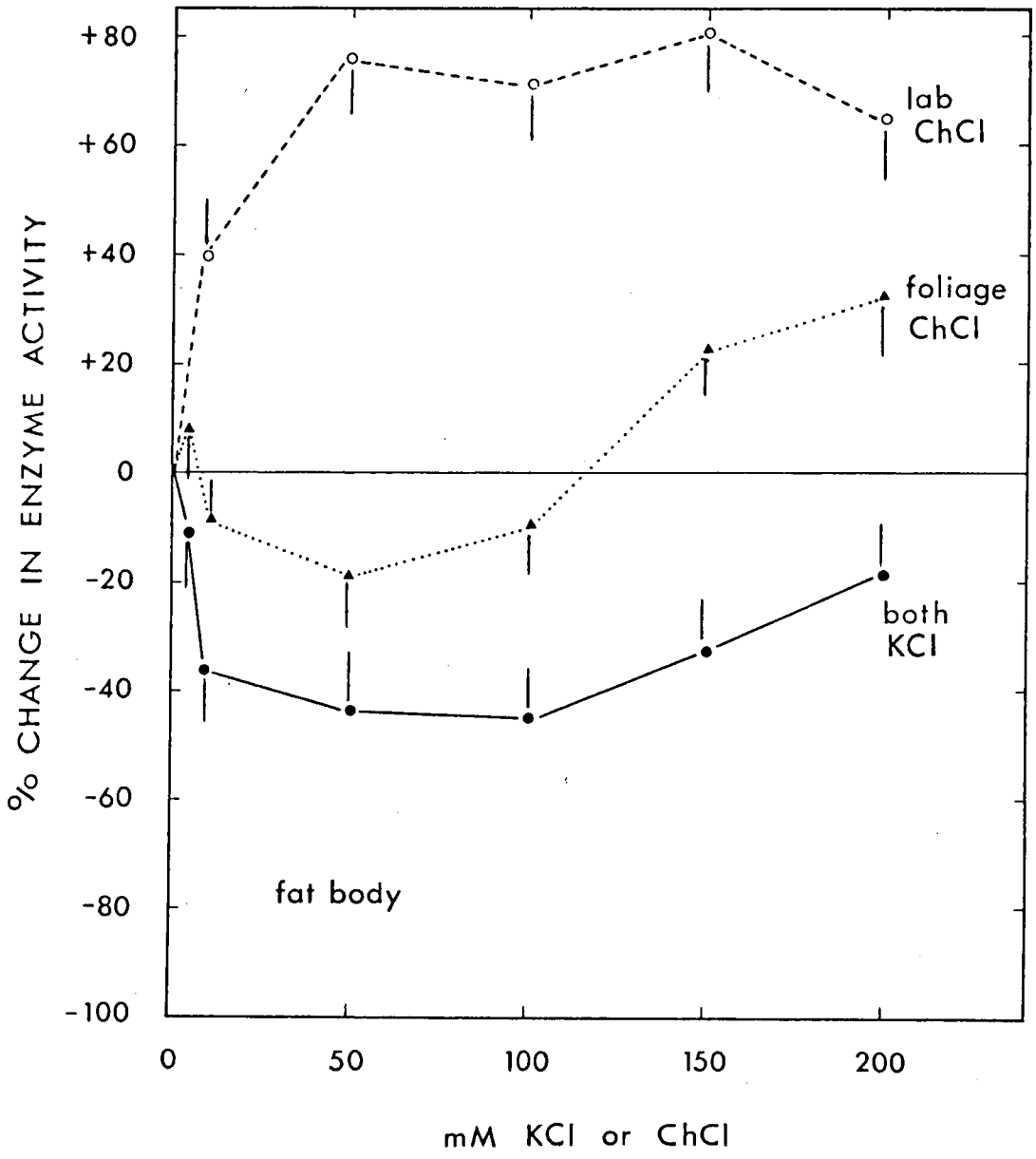


Figure 5.4. Effects of potassium chloride (KCl) and choline chloride (ChCl) on fifth instar fat body carbonic anhydrase from both synthetic diet and foliage reared silkmoths. The line marked both KCl indicates that the effects of KCl on foliage enzyme are not significantly different from those on lab enzyme.

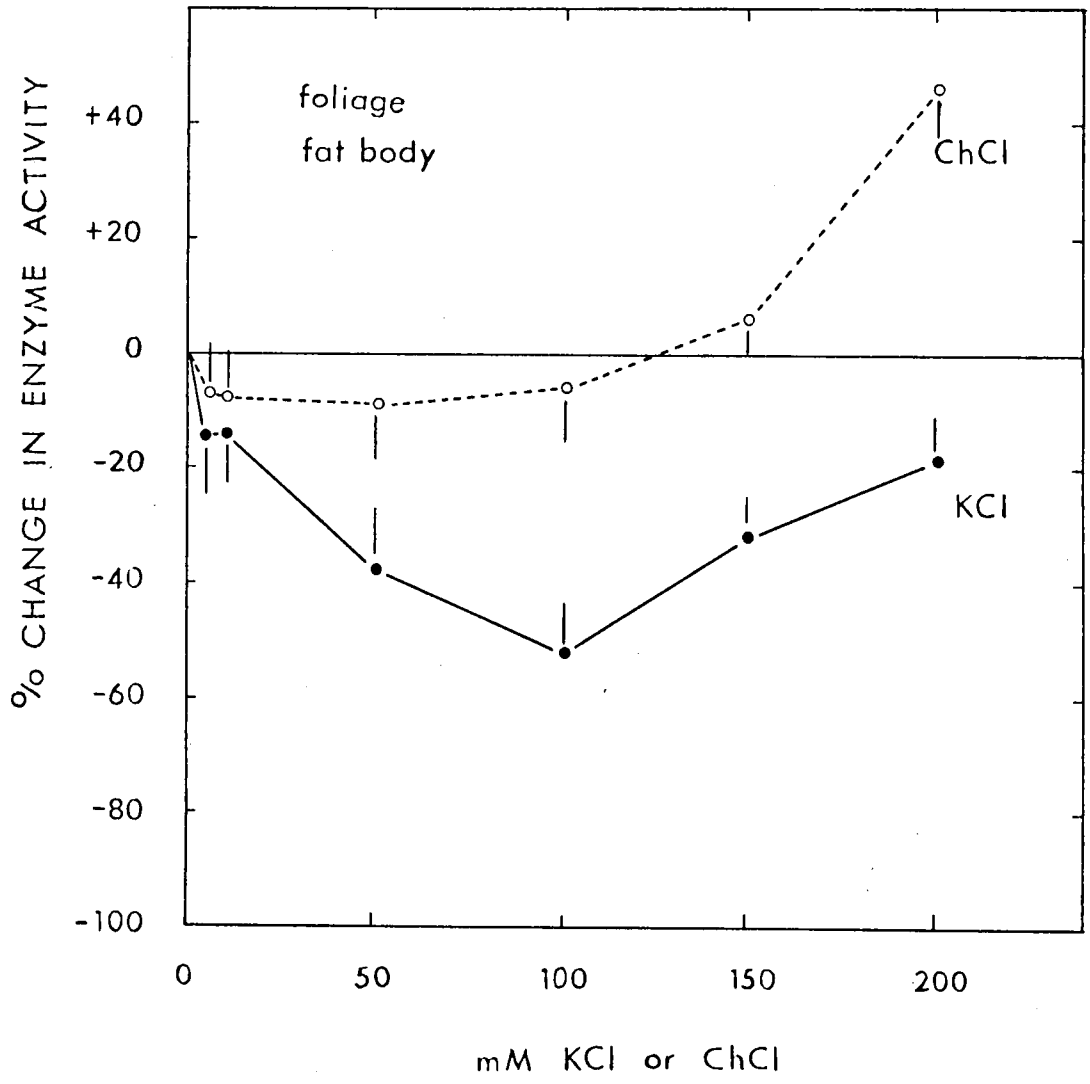


Figure 5.5. Effects of potassium chloride (KCl) and choline chloride (ChCl) on fat body carbonic anhydrase from foliage reared H. cecropia during moulting fluid elaboration.

KCl providing the greatest inhibitory effect (40%). The inhibition below 100 mM is due to an additive effect of K^+ and Cl^- and above 100 mM solely to K^+ inhibition. The effects of K^+ and Cl^- did not differ significantly from those seen with feeding fifth instar enzyme (compare Figure 5.4 with Figure 5.5).

The effects of KCl on lab reared fat body enzyme changes dramatically between the fifth instar and middle moultinf fluid stages (compare Figure 5.4 with Figure 5.6). Chloride, which had activated the enzyme 80% in the fifth instar, now inhibits the enzyme 60% during the middle moulting fluid stage (Figure 5.6).

Integument. Foliage and diet reared integumentary carbonic anhydrases are inhibited some 50 to 60% by KCl concentrations between 40 and 100 mM (Figure 5.7). In foliage animals, this inhibition is due to the combined effect of K^+ and Cl^- (see foliage ChCl line, Figure 5.7), while in lab reared animals it is entirely due to K^+ (i.e., Cl^- has no effect on the enzymatic activity, see lab ChCl line in Figure 5.7). During the middle moulting fluid stage, chloride no longer inhibits foliage carbonic anhydrase (Figure 5.8), which is now inhibited 20 to 30% primarily by potassium concentrations of 25 to 200 mM. In lab reared silkmoths, integumentary carbonic anhydrase changes between the fifth instar and the middle moulting fluid stage such that chloride, which was without effect on the enzyme in the feeding larva, becomes inhibitory (40%, Figure 5.9).

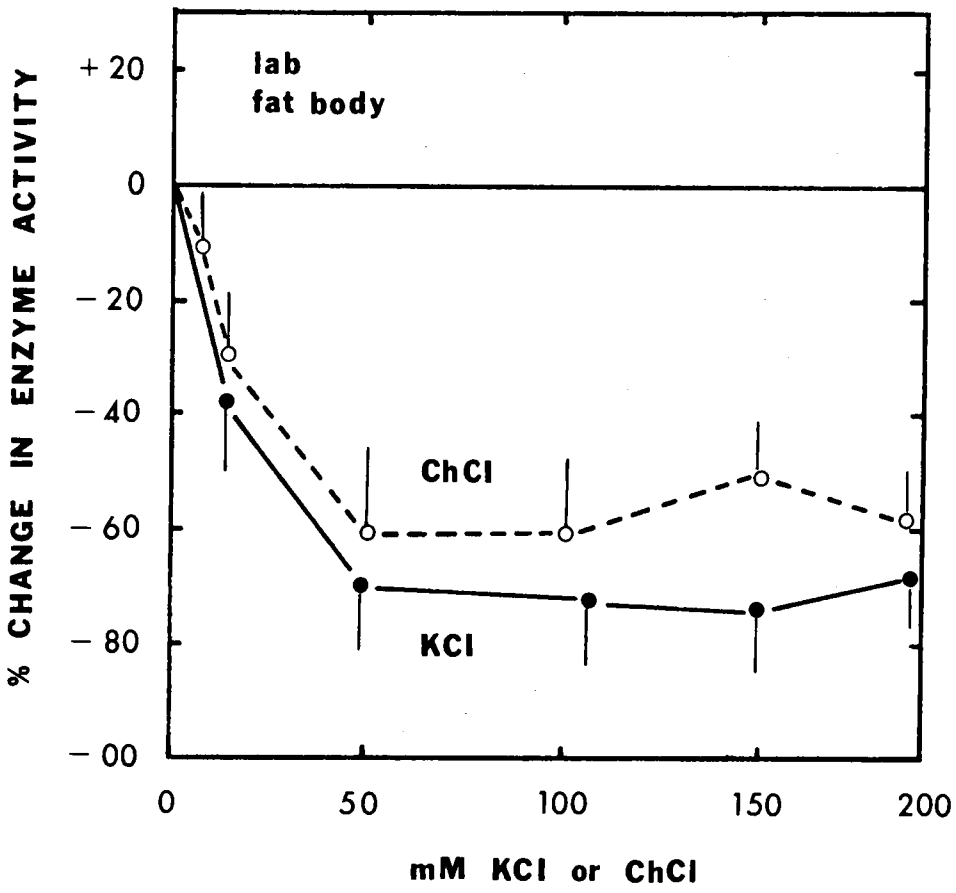


Figure 5.6. Effects of potassium chloride (KCl) and choline chloride (ChCl) on fat body carbonic anhydrase from synthetic diet reared H. cecropia during moulting fluid elaboration.

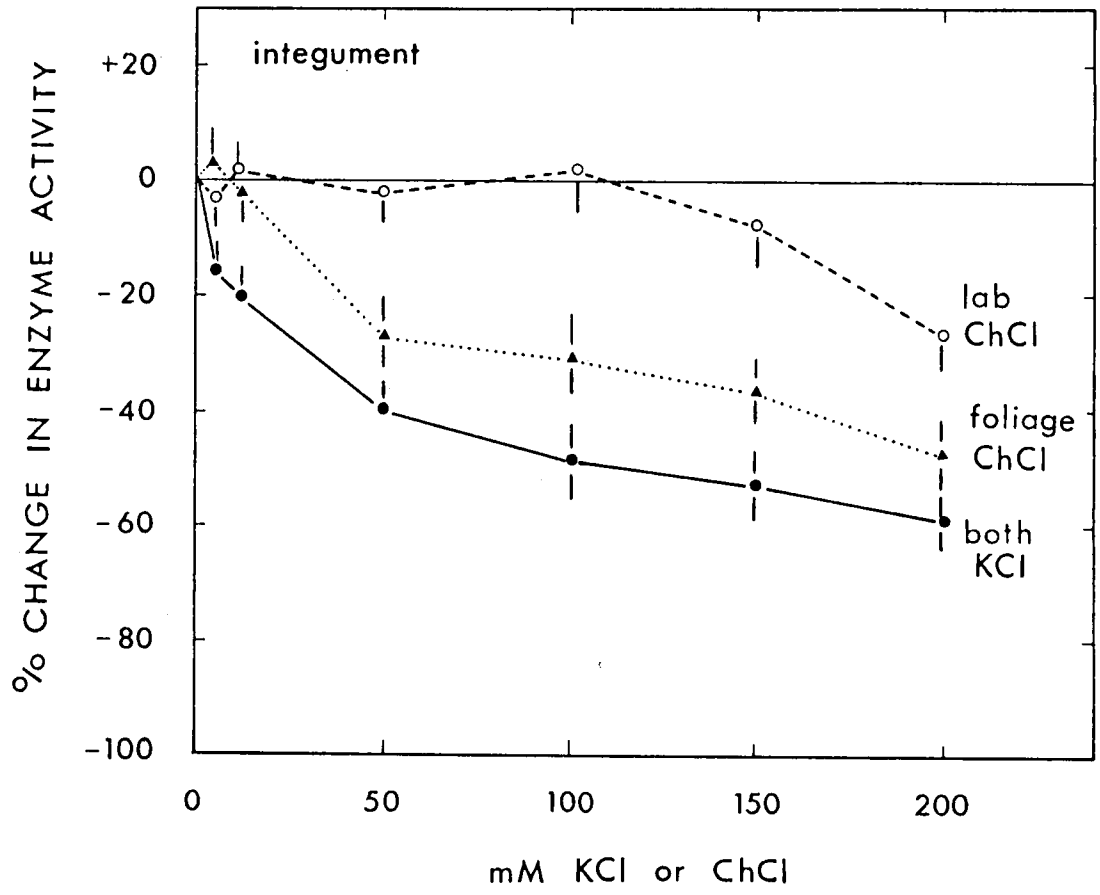


Figure 5.7. Effects of potassium chloride (KCl) and choline chloride (ChCl) on fifth instar integumentary carbonic anhydrase from both synthetic diet and foliage reared silkmoths. The line marked both KCl indicates the effects of KCl on foliage enzyme are not significantly different from those on synthetic diet enzyme.

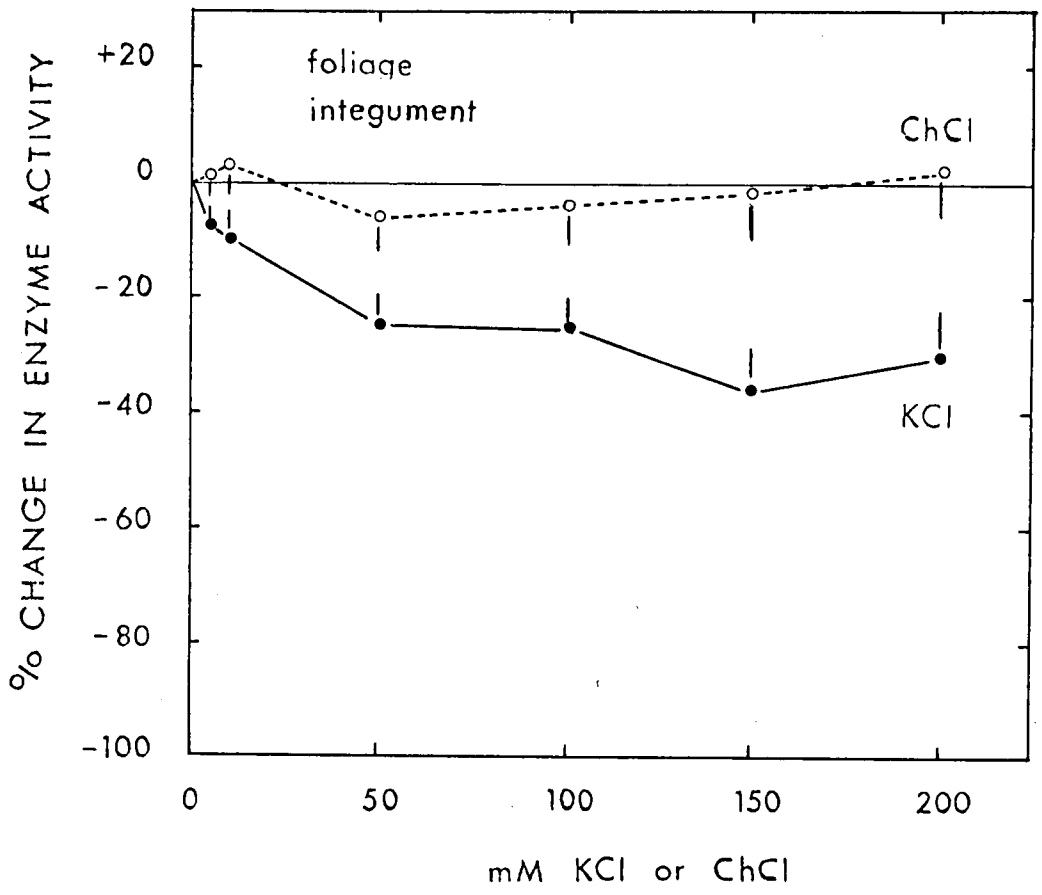


Figure 5.8. Effects of potassium chloride (KCl) and choline chloride (ChCl) on integumentary carbonic anhydrase activity from foliage reared H. cecropia during moulting fluid elaboration.

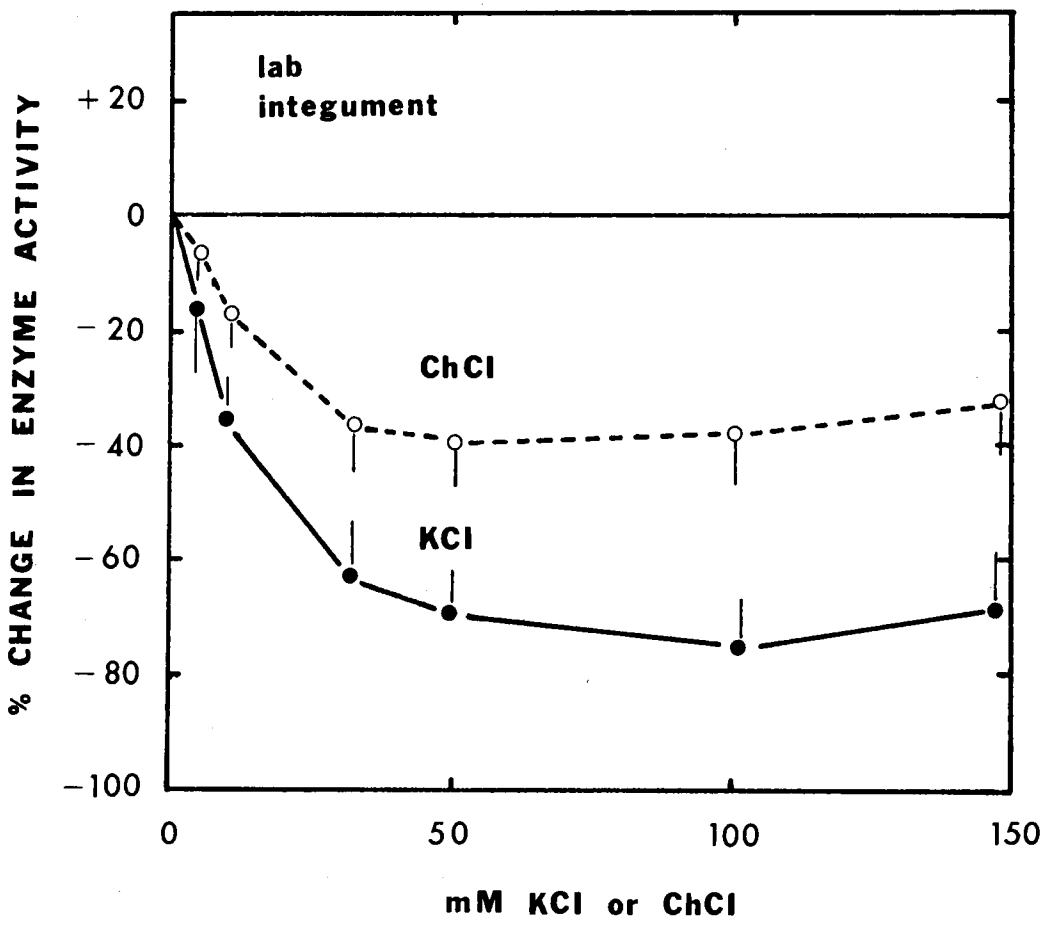


Figure 5.9. Effects of potassium chloride (KCl) and choline chloride (ChCl) on integumentary carbonic anhydrase activity from synthetic diet reared H. cecropia during moulting fluid elaboration.

Effects of K^+, Cs^+, Li^+, Rb^+ and Na^+

The effects of activation or inhibition of vertebrate carbonic anhydrases are dependent upon substrate (= CO_2) levels (Maren, 1971). I therefore examined the effects of CO_2 on insect carbonic anhydrase activity, and further the effects of alkali metals at different CO_2 levels on enzyme activity. Experiments were conducted with midgut, fat body and integument from foliage reared silkmoths at the fifth instar and middle moulting fluid stages. Separation of the effects of cations from anions is fundamental to this study. Therefore, the effects of alkali metal chloride salts are expressed as percentage change in enzymatic activity relative to choline chloride (Figure 5.10).

At a CO_2 concentration 0f 30.6 mM, midgut enzyme is slightly activated by 32 mM K^+ relative to Cl^- (see Figure 5.3, p. 114). However, lowering the CO_2 concentration to 15.6 mM causes these same cations to inhibit the enzyme by 40 to 50% (Figure 5.10).

In fifth instar fat body, at a CO_2 concentration of 30.6 mM, 32 mM K^+ inhibited the enzyme an additional 30% relative to Cl^- (Figure 5.4, p. 116). When the CO_2 concentration is halved (15.3 mM), the inhibitory effect of 32 mM K^+ (as well as all other cations studied) more than doubled (-70%, see Figure 5.10). Surprisingly, no significant differences in response is observed when middle moulting fat body is employed (compare Figure 5.5, p. 117, with the right hand portion of Figure 5.10).

Integumentary carbonic anhydrase from the fifth larval instar, in the presence of 30.6 mM CO_2, in inhibited by an additional 10% at 32 mM

Figure 5.10. Effects of potassium chloride (K^+), cessium chloride (Cs^+), lithium chloride (Li^+), rubidium chloride (Rb^+) and sodium chloride (Na^+), relative to choline chloride, on midgut, fat body and integumentary carbonic anhydrases during the fifth instar (left portion), and on fat body and integumentary carbonic anhydrases during moulting fluid stages (right portion) of foliage reared H. cecropia. The results are expressed as percentage change in enzyme activity relative to the effects of choline chloride. All salts were used at a concentration of 32 mM, and the CO_2 concentration was 15.3 mM (see Methods section).

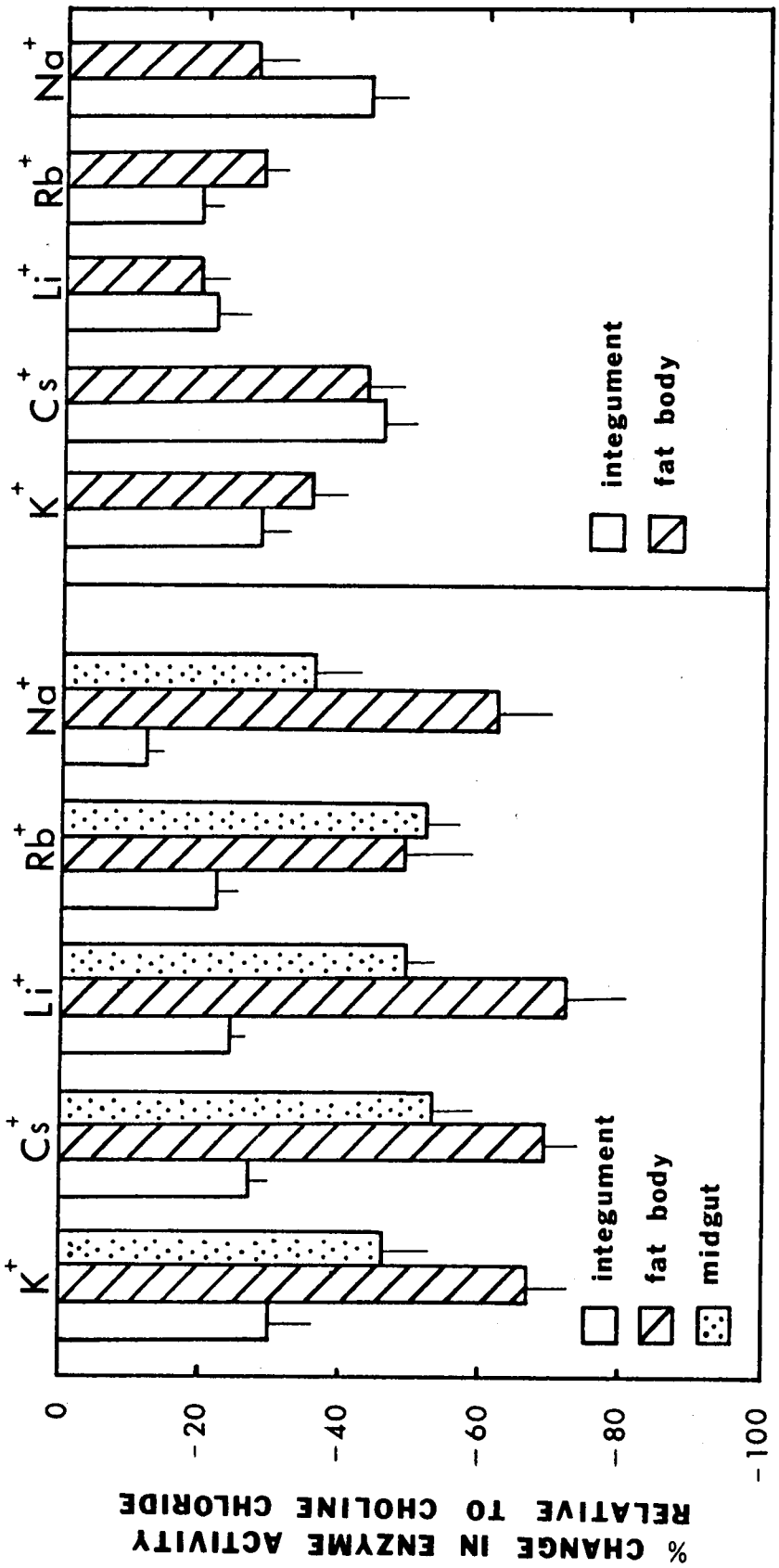

% CHANGE IN ENZYME ACTIVITY RELATIVE TO CHOLINE CHLORIDE
0
-20
-40
-60
-80
-100
K+
Cs+
Li+
Rb+
Na+
integument
fat body
midgut
FEEDING FIFTH INSTAR
K+
Cs+
Li+
Rb+
Na+
integument
fat body
MOULTING FLUID

Figure 5.10

K^+ relative to Cl^- (Figure 5.7, p. 120). When the CO_2 concentration was reduced to 15.3 mM, all alkali metal cations studied (except Na) inhibited the enzyme by an additional 25 to 35% relative to Cl^- inhibition alone (Figure 5.10). At the middle moulting fluid stage, 32 mM K^+ inhibited the enzyme by an additional 15% relative to Cl^- (CO_2 was 30.6 mM, Figure 5.8, p. 121). At a CO_2 concentration of 15.3 mM, the alkali metal cations inhibited the enzyme by an additional 20 to 45% relative to Cl^- alone (Figure 5.10), with Na^+ and Cs^+ showing significantly increased capacities to alter the enzymatic activity (especially compare the Na^+ effects in the right and left hand portions of Figure 5.10).

IV. DISCUSSION

Midgut

Studies on the effects of acetazolamide, K^+, other alkali metal and Cl^- demonstrate that the response of midgut carbonic anhydrase differs depending upon diet. The ease of interconverting these enzymes attests to the lack of some factor (as yet undetermined) in the diet of lab reared animals in whose absence K^+ inhibits rather than activates the midgut enzyme (see Figure 5.3, p. 114). This situation is analogous to that described in H. cecropia by Blankenmeyer and Harvey (1978) wherein electrical coupling of goblet cells with columnar cells occurs only in midgut tissues from lab reared animals. This phenomenon does not occur in vivo in midgut of foliage reared silkworms. It appears, therefore, that through the use of different rearing techniques, one may be able to

answer questions about enzyme production and regulation, and about trans-cellular ion transport. Whether the same factor is responsible for causing electrical coupling and transformation of the midgut carbonic anhydrase activities is unknown.

The high levels of carbonic anhydrase seen in midgut tissue from feeding fifth animals are very likely involved in the electrogenic transport of K^+ by the gut (Harvey and Nedergaard, 1964). In the stomach of vertebrates, carbonic anhydrase is involved in the secretion of H^+ into the lumen of the stomach, and an analogous situation of bicarbonate transport into midgut lumenal contents is not unlikely. Ethoxyzolamide (a potent inhibitor of carbonic anhydrase) has been shown to inhibit the short-circuit current of the foliage integument by 36%, while sodium sulfide (a noncompetitive inhibitor of carbonic anhydrase) completely inhibited the current generated by the silkmoth midgut (Haskell, Clemons and Harvey, 1965). The short-circuit current measured in isolated desert locust, Schistocerca gregaria, rectum is also inhibited by acetazolamide (Williams, et al., 1978).

How does inhibition of carbonic anhydrase cause a reduction in the current generated by this particular tissue? The following model is proposed.

1. Inhibition of carbonic anhydrase could reduce the number of non-electrogenic HCO_3^- equivalents available for co-transport with electrogenically transported K^+ such that active transport of K^+ is forcibly reduced.

2. The reduced availability of bicarbonate following acetazolamide treatment could interfere with the activation of a bicarbonate stimulated ATPase, to the detriment of the short-circuit current.

An ATPase with alkaline pH optium, that is stimulated by HCO_3^- has been demonstrated in H. cecropia midgut (Turbeck, Nedergaard and Kruse, 1968). Activation of enzymes by HCO_3^- is not unique to insects, since Merryfield (1979) has found that HCO_3^- activates rat liver p-enolpyruvate carboxy kinase ferroactivator, while Rudolph (1979) has found a dialyzable factor, possible HCO_3^- which can interact with the cyclic AMP-dependent transport mechanism in frog erythrocytes.

The present study has defined some of the basic parameters and problems involved in the study of insect carbonic anhydrase. I have not only shown that hemolymph is devoid of carbonic anhydrase activity (see also Buck and Friedman, 1958), but I have also demonstrated that muscle, cuticle, silk gland and moulting fluid are without carbonic anhydrase activity at any stage studied. Only midgut from foliage reared animals was found to be contaminated with non-insect carbonic anhydrase activity. Contamination of 500 mg of midgut tissue with as little as 10 micro-liters of ingested leaf gut content fluid will introduce an error of up to 50% in the measured carbonic anhydrase activity.

The concentration of CO_2 greatly influences the extent of cation or anion activation or inhibition (Figure 5.10). Adding to this complexity is an inability to fully separate alkali metal effects in the presence of halide anions. However, this problem is not unique to the insect enzyme as I have shown it to be troubling with mammalian enzymes as well (Chapter 6).

Fat Body

The initial decremental increase in fat body carbonic anhydrase activity noted between the fifth instar and initiation of spinning (Table 5.2, p. 104) in foliage animals results from an approximate doubling of the specific activity of the enzyme and not from an increase in total tissue mass (.9 gms at feeding fifth versus 1.1 gms at initiation of spinning). The second increase noted between spin + 2 days and the day of apolysis is also due to 1.7 fold increase in the specific activity (Table 5.1, p. 103) of the enzyme, since total fat body weight is the same (1.1 gms) at both stages. A third increase (25%) in total enzymatic activity occurs between apolysis + 1 day and apolysis + 2 days and it is due to a combination of increasing fat body tissue (16%) and increasing specific activity of the enzyme (11%, see Tables 5.1 and 5.2). Thereafter, total carbonic anhydrase activity in foliage fat body declines from apolysis + 2 days until the larval-pupal ecdysis despite a 30% increase in fat body weight. The 75% reduction in enzymatic activity is due either to an absolute loss of enzyme or to masking of the enzyme present (Figure 5.1, p. 110).

The most obvious difference in enzymatic activity between foliage and snythetic diet reared fat bodies occurs during the period from apolysis + 4 days through the larval-pupal ecdysis. Carbonic anhydrase in lab reared fat body more than triples during this period, which contrasts with foliage enzyme which falls by 67% over this same period.

How the type (e.g., anion sensitive) and quantity of fat body carbonic anhydrase is regulated during development is not known. In

this report I show that the two carbonic anhydrases are differentially affected by Cl^- (see Figures 5.4 through 5.6, pp. 116, 117, and 119). I also believe that this differential response to chloride is a part of the overall regulatory process, since fat body from synthetic diet insects, which is inhibited by Cl^-, may contain significantly higher levels of intercellular Cl^- than fat body from foliage reared animals (see Chapter 3). These two observations would lead one to expect a reduction in the total *in vivo* exzymatic activity in lab fat body, yet the opposite is seen *in vitro* (Table 5.4) where Cl^- effects are virtually eliminated. A third, and deciding, factor in this argument is that foliage reared *H. cecropia* have 3 to 5 times more HCO_3^- in hemolymph than do synthetic diet reared animals during the moulting fluid stages (see Chapter 3). Assuming that carbonic anhydrase activity in fat body is responsible for HCO_3^- present in the hemolymph (a reasonable argument based on the lack of enzymatic activity in other tissues) I propose that there is a positive feedback loop between HCO_3^- and carbonic anhydrase activity. With this model, a vicious cycle develops in synthetic diet *H. cecropia*. The insect's fat body enzyme is so inhibited by Cl^- *in vivo* that hemolymph HCO_3^- levels remain extremely low (5 mM versus 30+ mM in foliage animals), because increased enzymatic activity due to low HCO_3^- cannot be expressed in the presence of high intracellular Cl^- *in vivo*. When assayed *in vitro*, the carbonic anhydrase is removed from its high *in vivo* Cl^- environment and increased activity can be expressed.

This problem is not encountered by foliage animals for two reasons. Firstly, Cl^- does not affect the enzyme at the moulting fluid stage

(see Figure 5.5, p. 117), and secondly, intracellular Cl^- levels are low relative to those animals reared on synthetic diet. Thus, foliage reared silkmoths are capable of producing higher levels of HCO_3^- in hemolymph than diet reared insects, despite similar levels of activity measured under in vitro conditions.

Integument

The decline in total carbonic anhydrase in integument between the feeding larva and the day of spin in both foliage (Table 5.2, p. 104) and synthetic diet (Table 5.4, p. 106) silkworms is due to a decrease (about 27%) in total integument weight as well as a decline (10 to 25%) in the absolute activity of the enzyme. Despite a continued weight loss in integument (12%), total integumentary carbonic anhydrase activity (Tables 5.2 and 5.4) remains relatively constant in both rearing groups, until apolysis. At apolysis there is an unexplained doubling of activity in diet reared animals only (see Figure 5.1, p. 110), with diet reared integument possessing only half the activity observed in foliage tissue between apolysis and early moulting fluid. A second diet specific increase in integument enzyme activity, from synthetic diet insects only, occurs at early moulting fluid (see Table 5.2 and Figure 5.1). Activity declines in integument from both diets between early moulting and the larval-pupal ecdysis. The absence of these two periods of increased activity in foliage animals, coupled with the differential effects of Cl^- at the feeding fifth stage (Figure 5.7, p. 120), and both K^+ and Cl^- at moulting fluid stages (compare Figure

5.8 with 5.9, pp. 121-122), leads me to conclude that the two enzymes (lab versus foliage) are fundamentally different.

In an analysis of H. cecropia hemolymph and moulting fluid (Chapter 3), synthetic diet reared animals were found to have half the moulting fluid bicarbonate levels of foliage animals. Based on this study, the differences in the regulation of integumentary carbonic anhydrase between animals reared on the two diets is responsible for the concentration inequality. Measured in vitro, carbonic anhydrase in integument of synthetic diet reared animals at the time of moulting fluid elaboration is sufficient to produce HCO_3^- at rates comparable to that produced by foliage integument. Upon closer examination, the synthetic diet enzyme is twice as sensitive to K^+ and Cl^- as the foliage enzyme (compare Figure 5.8 with 5.9). I believe that cation and anion inhibition mask the activity in vivo, such that the lab integument is capable of producing only half the number of moulting fluid HCO_3^- equivalents present in foliage animals (Chapter 3). If this proposal is correct, then the prediction that HCO_3^- accompanies actively transported K^+ during moulting fluid secretion (Jungreis, 1979; Jungreis and Harvey, 1975) could certainly be proven by comparing lab and foliage reared animals.

CHAPTER 6

COMPARATIVE PROPERTIES OF MAMMALIAN AND INSECT CARBONIC ANHYDRASES: EFFECTS OF POTASSIUM AND CHLORIDE ON THE RATE OF CARBON DIOXIDE HYDRATION

I. INTRODUCTION

I have been studying the involvement of insect carbonic anhydrase (C.A.) (E.C. 4.2.1.1) in the formation of Lepidopteran larval-pupal moulting fluid, a potassium bicarbonate salt solution (Jungreis, 1974, 1978; Johnston and Jungreis, 1977). In my assays, a commercially prepared enzyme with known activity, bovine C.A., was routinely employed as a standard. Several studies describing the inhibitory effects of halide anions on bovine C.A. catalysed carbon dioxide (CO_2) hydration have recently been reported (Maren, Rayburn and Liddell, 1976; Koenig and Brown, 1976; Pocker and Tanka, 1978). In these latter studies, potassium halides were routinely employed, yet control studies on the effects of halide anions in the absence of potassium (or sodium) have not been reported. In my studies on insect C.A., the effects of potassium and halide anions could be separated readily, and proved to be different from the effects on the bovine enzyme (Turbock and Foder, 1970; Johnston and Jungreis, 1978; in preparation). I therefore undertook a more systematic study of the effects of potassium and chloride on carbonic anhydrase catalysed CO_2 hydration by mammalian enzymes.

I have now examined 8 mammalian and 3 insect C.A.'s at a variety of CO_2, potassium and chloride concentrations. All of the mammalian enzymes were markedly inhibited by both potassium and chloride, with the extent of inhibition greater with KCl than with choline chloride (ChCl). The insect enzymes were slightly inhibited by ChCl, but greatly stimulated by KCl. I propose that the regulation of carbonic anhydrase catalysed CO_2 hydration by K^+ is fundamentally different in insects and mammals.

II. MATERIALS AND METHODS

Enzyme Preparation

Tobacco hornworms, Manduca sexta, were reared following procedures described in Ely and Jungreis (1977). Feeding fifth instar larvae (6-10 g) were chilled in crushed ice for 30 minutes. An incision was then made along the dorsum of the abdomen to facilitate the removal of hemolymph. Animals were then placed on a Bailey TISSU-FREEZ cold plate (Bailey Instruments, Saddlebrook, New Jersey) maintained at 0°C, and fat, body, midgut and integumentary epithelium were dissected free from other tissues, or from the cuticle proper. Excised tissues were then blotted on weighing paper, weighed to the nearest milligram, transferred to vials containing 9 volumes of 0.015 M Hepes Buffer (pH 8.3) to which were added several crystals of phenylthiourea to prevent melanization, and sonicated for 30 seconds in crushed ice with a Biosonik Sonicator (Bromwill Scientific Corporation, Rochester, New York). Sonicates were then centrifuged for 2 minutes in a clinical centrifuge, and the

supernatant fractions decanted into test tubes. All (100%) of the C.A. activity is recovered in the supernatant fraction.

Mammalian enzymes: Bovine, Bovine A, Bovine B, Dog, Human A, Human B, Human C and Rabbit Carbonic Anhydrase were all purchased from Sigma Chemical Corporation (St. Louis, MO).

Enzyme Assay

Carbonic anhydrase activity can be measured as a function of the rate of C.A. catalysed CO_2 hydration by a modification of the electrometric method of Wilbur and Anderson (1948). The assay solution is composed of 6 ml of 0.015 M Hepes Buffer (pH 8.3) at 3°C and 0.10-0.30 ml of enzyme solution to which are added 4 ml of CO_2 saturated water (3°C). We have defined one Wilbur-Anderson (W-A) unit as that quantity of enzyme that will cause this solution to go from pH 8.3 to pH 6.8 in one minute at 3°C. An attempt was made to always have 2 W-A units of enzyme activity present at the onset of each assay, so as to facilitate the comparison of inhibitory effects of potassium and chloride on the different mammalian and insect enzymes.

The type of inhibition that chloride causes during bovine C.A. catalysed CO_2 hydration is reputed to be kinetically noncompetitive (Maren, Rayburn and Liddell, 1976; Pocker and Tanaka, 1978). However, since assay conditions employed in these reports always involved potassium salts, we undertook to reexamine in the absence of K^+ the effects of chloride on bovine C.A. catalysed CO_2 hydration. Rates of C.A. catalysed CO_2 hydration were studied at CO_2 levels of 0.0076, 0.0154, 0.0228 and 0.0308 M in the presence or absence of 0.005-0.15 M

potassium or choline chloride. The definition of a W-A enzyme unit given above is unsatisfactory at CO_2 levels below 0.02 M. Therefore, the W-A unit was modified as follows: At CO_2 concentrations of 0.0154 and 0.0076 M, one W-A unit is defined as that activity which will cause the pH to drop from 8.3 to 7.3, and 8.03 to 7.8, respectively, in one minute.

III. RESULTS

Insect Carbonic Anhydrases

In the absence of exogenous chloride salts and at a CO_2 concentration of 0.0308 M, approximately 2 W-A units of C.A. were assayed from fat body, midgut and integumentary epithelium. Cuticle and the musculature underlying the integumentary epithelium are without C.A. activity at this stage in development.

Effects of choline chloride. With integument, addition of 5-30 mM ChCl caused an approximately 10% reduction in activity, whereas no loss was noted at 50-150 mM ChCl (Figure 6.1). This result is in contrast to midgut which exhibits a 40% increase in activity in 5 mM ChCl, a 10% increase in 10 mM ChCl and a 10% reduction in 30 mM ChCl. Concentrations of ChCl in excess of 50 mM stimulate midgut C.A. until a maximal 40% increase in achieved in 150 mM ChCl. A third pattern of response is observed in fat body. Here, C.A. exhibits the maximal inhibition of 30% between 10-50 mM ChCl, but no inhibition at ChCl concentrations of 100-150 mM.

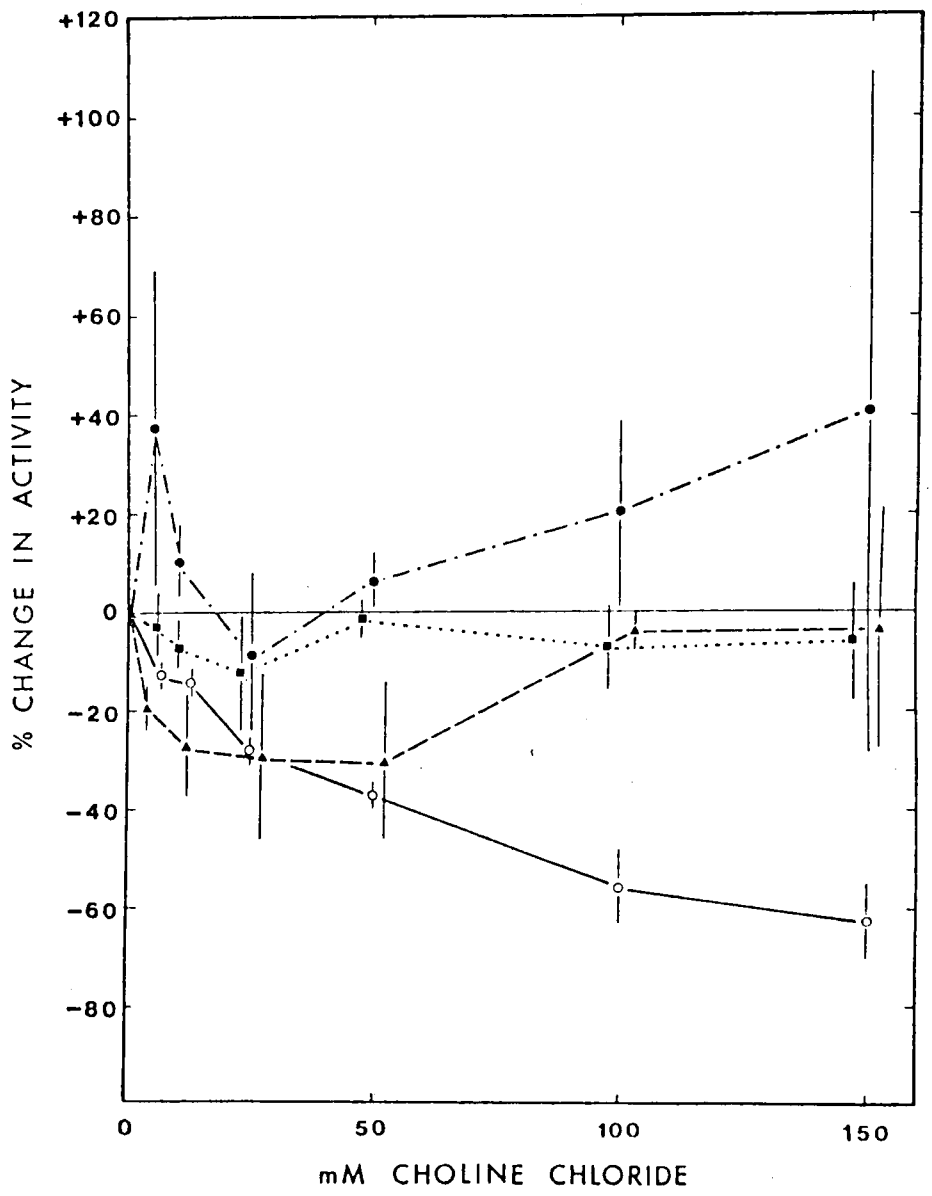


Figure 6.1. Effects of choline chloride on bovine and insect carbonic anhydrase catalysed CO_2 hydration. ● = midgut, ▲ = fat body, ■ = integument, O = bovine.

These responses of insect C.A. are contrasted with those of bovine, which exhibits a graded loss of activity with increasing concentrations of ChCl.

Effects of potassium chloride. Although ChCl caused a slight inhibition of integumentary epithelial C.A., KCl at concentrations between 5-15 mM is without effect except at 30 mM, where a 15% increase in activity is noted (Figure 6.2). The responses of midgut and fat body C.A. to KCl are markedly different from their responses to ChCl. Both enzymes exhibit dramatic increases in activity between 5-30 mM KCl, with a sustained 60% activation noted between 30-100 mM. C.A. activity continues to increase as the concentration of KCl reaches 150 mM, with midgut exhibiting a maximal 100% increase in activity. Increases in potassium activation do not increase beyond 150 mM, since C.A. activity in 200 mM KCl is reduced for both midgut and fat body relative to 100 mM activity levels.

The response of bovine C.A. to KCl is opposite that observed for insect carbonic anhydrases. The bovine enzyme exhibits a precipitous 50% decline in C.A. activity even at 10 mM KCl. This reduced level of activity is further depressed by 1/5th as the concentration of KCl increases to 100-150 mM.

Mammalian Carbonic Anhydrases

Mammalian carbonic anhydrases were measured at CO_2 concentrations between 0.0076-0.0308 M and at concentrations of KCl of ChCl between 10-100 mM. All eight mammalian enzymes studied were inhibited by these

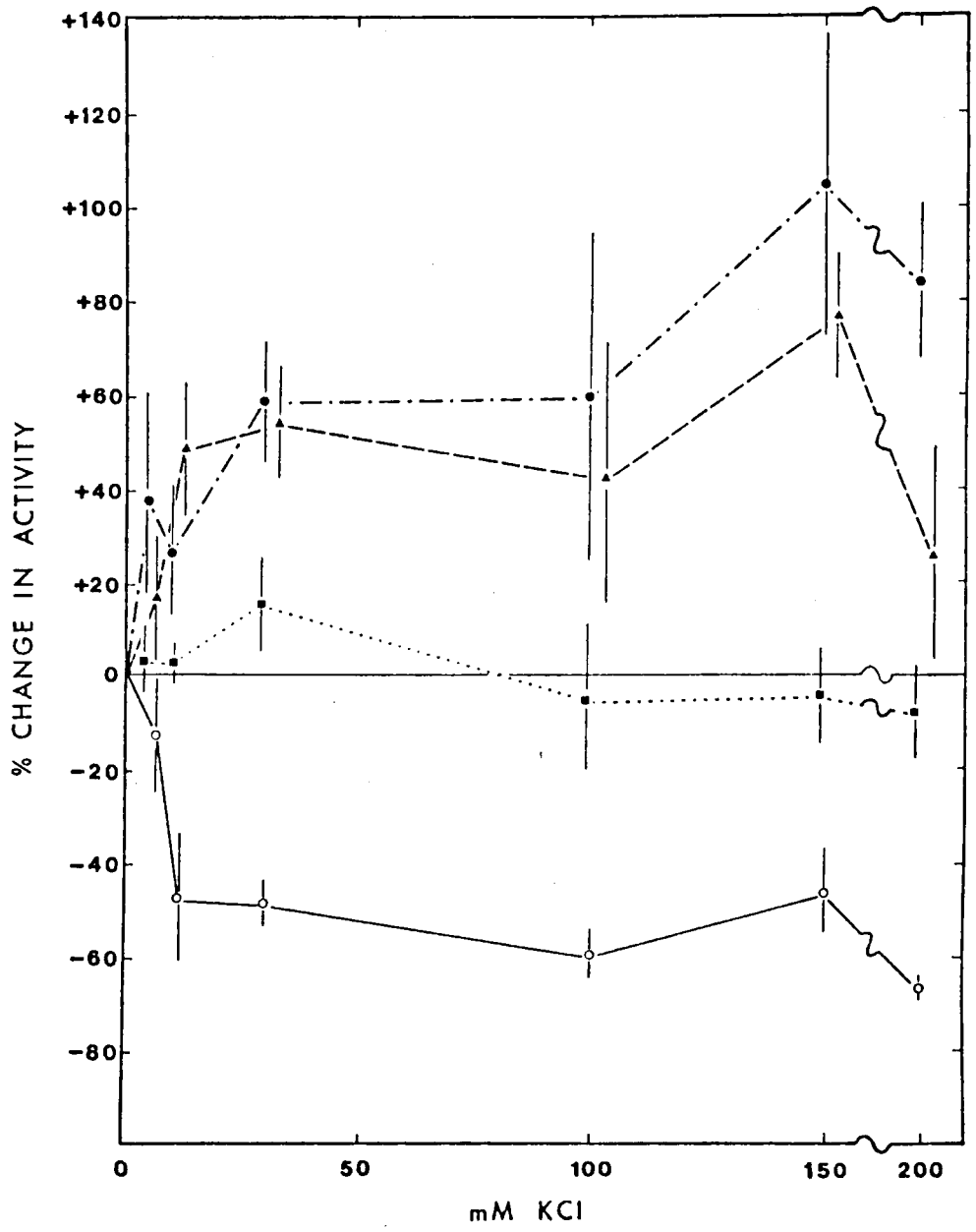


Figure 6.2. The effects of potassium chloride on bovine and insect carbonic anhydrase catalysed CO_2 hydration. ● = midgut, ▲ = fat body, ■ = integument, O = bovine.

chloride salts regardless of the concentration of CO_2 (Table 6.1). Potassium chloride is always more inhibitory than choline chloride at CO_2 concentrations of 0.015 M or less. At CO_2 levels of 0.0308 M, for five of these eight mammalian enzymes, 10 mM ChCl was as inhibitory as the respective KCl solution, but at higher salt concentrations (e.g., 100 mM) KCl is always more inhibitory than ChCl ($p < 0.01$). The order of sensitivity to chloride salts at 0.0152 M CO_2 is (in decreasing order) Human B > Human A > Bovine A > Bovine B > Dog > Bovine > Rabbit > Human C. The order of sensitivity to chloride salts at 0.0308 M CO_2 is (in decreasing order) Human B > Human A > Bovine > Bovine A > Bovine B > Human C > Dog > Rabbit.

The data in Table 6.1 clearly demonstrate the presence of an effect of K^+ that is additive to that of the Cl^- inhibition reported by Maren, Rayburn and Liddell (1976) and Pocker and Tanaka (1978). The study of Cl^- inhibition of mammalian C.A. catalysed CO_2 hydration can be studied best with the Human B enzyme (as was done in the studies of Maren, Rayburn and Liddell, 1976), since it exhibits maximum halide and minimal K^+ sensitivity. However, since Pocker and Tanaka (1978) used bovine C.A. to prove that Cl^- is a kinetically noncompetitive inhibitor of C.A. catalysed CO_2 hydration, we examined the kinetics of K^+ and Cl^- inhibition with this enzyme.

Two W-A units of C.A. were employed at CO_2 concentrations of 0.0076 and 0.0226 M. Activity was then measured in the presence of 50 or 100 mM KCl or ChCl. In the presence of K^+, Cl^- noncompetitively inhibits C.A. catalysed CO_2 hydration, in agreement with earlier reports (Maren,

TABLE 6.1. Carbonic anhydrase activity remaining at 0.0152 or 0.0308 M CO_2 levels following addition of 0.010 or 0.100 M KCl or choline chloride. Initial activity in the absence of added sale was 2.00 W-A units.

	Mammalia Enzymes							
	Human C	Bovine	Rabbit	Dog	Bovine A	Bovine B	Human A	Human B
15.2 mM CO_2								
10 mM KCl	1.46	1.05	0.94	0.90	0.87	0.79	0.63	0.47
10 mM Choline Chloride	1.73	1.47	1.25	1.20	1.41	1.52	0.67	0.54
100 mM KCl	0.76	0.34	0.66	0.35	0.25	0.25	0.00	0.00
100 mM Choline Chloride	1.01	0.59	0.95	0.87	0.52	0.53	0.00	0.00
KCl / Choline Chloride Ratio 10 mM	0.84	0.71	0.75	0.76	0.52	0.94	0.94	0.85
KCl / Choline Chloride Ratio 100 mM	0.75	0.57	0.70	0.40	0.48	0.47	--	--
30.8 mM CO_2								
10 mM KCl	1.32	1.58	1.75	1.63	1.63	1.64	0.92	0.81
10 mM Choline Chloride	1.76	1.50	1.64	1.64	1.53	1.72	1.11	0.81
100 mM KCl	1.03	0.71	1.36	0.99	0.93	0.90	0.17	0.16
100 mM Choline Chloride	1.31	1.05	1.68	1.34	1.11	1.16	0.37	0.23
KCl / Choline Chloride Ratio 10 mM	0.75	1.05	1.07	0.99	1.07	0.95	0.83	1.00
KCl / Choline Chloride Ratio 100 mM	0.78	0.68	0.81	0.74	0.84	0.77	0.46	0.70

Rayburn and Liddell, 1976; Pocker and Tanaka, 1978) (Figure 6.3, see Hanes, 1932; Christensen and Palmer, 1974, for the derivation of the Woolf plot). However, when choline was substituted for K^+, Cl^- acts as a competitive inhibitor (Figure 6.4), as was predicted by Koenig and Brown, 1976). Thus, in the absence of K^+, Cl^- is a competitive inhibitor of both CO_2 hydration and HCO_3 dehydration (see Pocker and Tanaka, 1978; Koenig and Brown, 1976), while in the presence of K^+, Cl^- is a noncompetitive inhibitor of CO_2 in hydration (see Pocker and Tanaka, 1978; Maren, Rayburn and Liddell, 1976). Thus, one sees how the failure to employ suitable controls during kinetic analysis can lead to erroneous conclusions.

IV. DISCUSSION

The mechanism of action of mammalian carbonic anhydrase catalysed CO_2 hydration can be inferred from the models of Riepe and Wang (1967) and Pocker and Stone (1967) (Figure 6.5). Carbon dioxide hydration involves a nucleophilic attack of bound water in the catalytic site to produce a hydroxyl group. The hydroxyl group then attacks a CO_2 molecule to produce carbonic acid (H_2CO_3). Formation of hydroxyl ion from water is facilitated by zinc (Zn^{++}) metal ion bound at the catalytic site, and by protonation of a histidyl associated imidazole side chain adjacent to the catalytic site. Based on this model of carbonic anhydrase action, halide anions would displace hydroxyl ions at the catalytic site. The extent of displacement (at constant pH) would then be dependent upon the halide anion concentration. The kinetics of

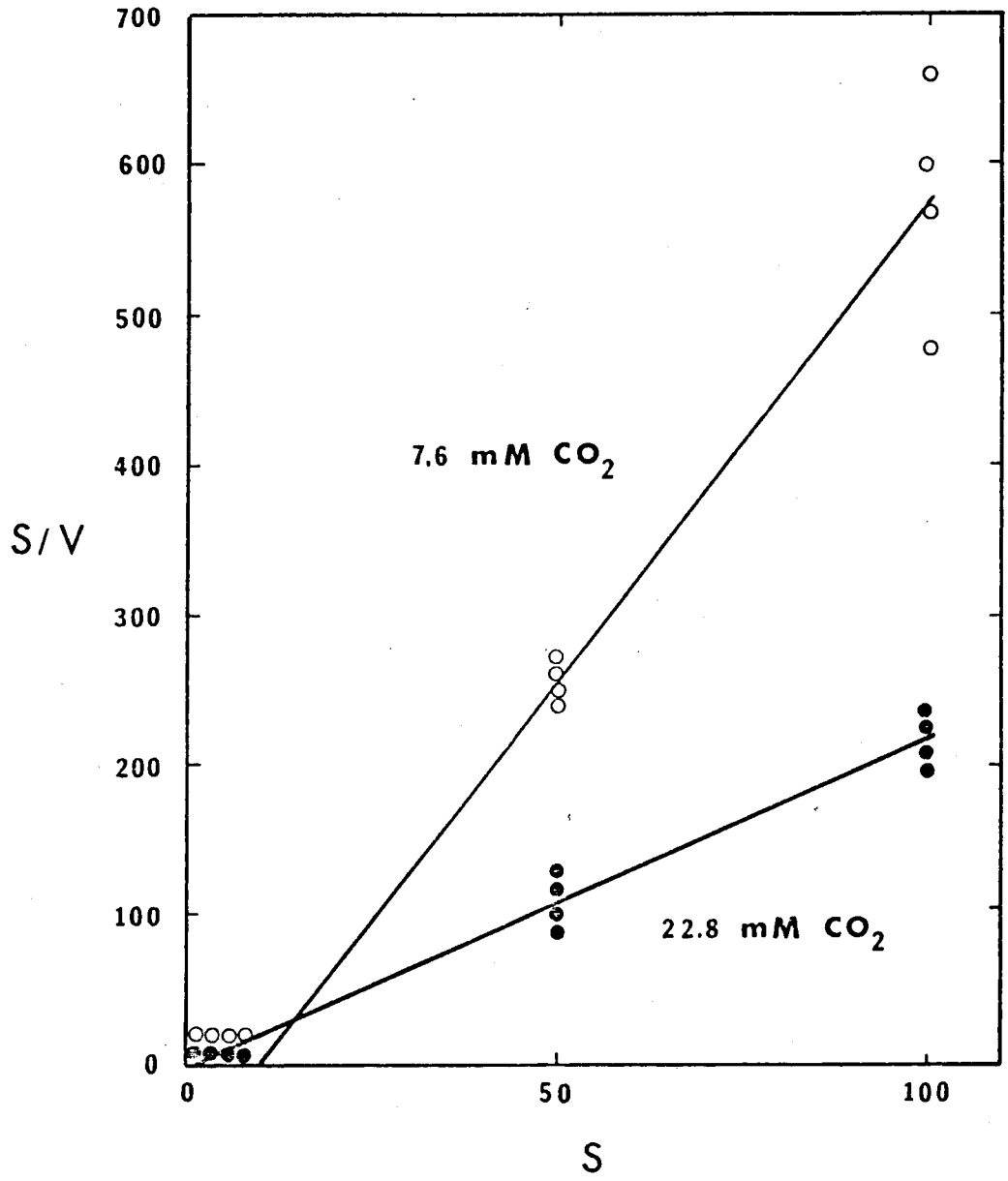


Figure 6.3. Noncompetitive inhibition of bovine carbonic anhydrase catalysed CO_2 hydration in the presence of potassium chloride salts. Open circles 0.0076 M CO_2: Y = 6.41 X - 62.3; Shaded circles 0.0228 M CO_2: Y = 2.23 X - 443.

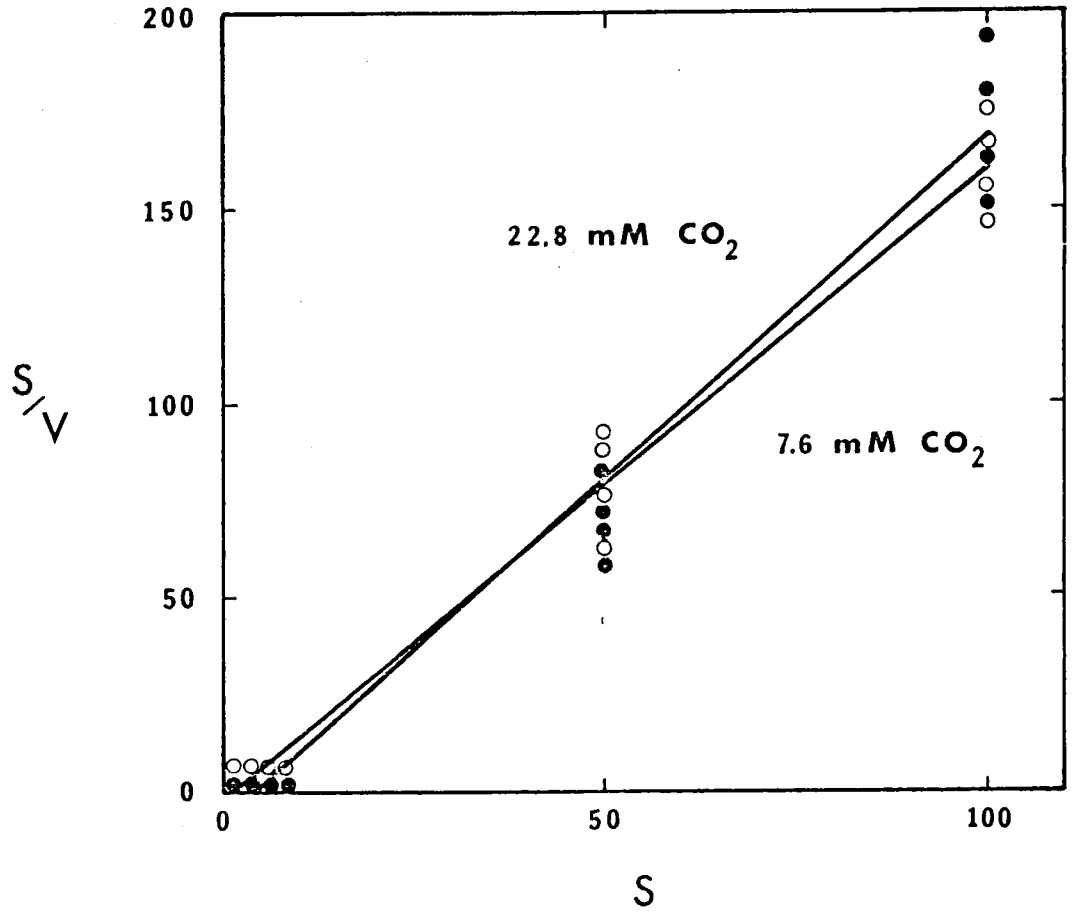


Figure 6.4. Competitive inhibition of bovine carbonic anhydrase catalysed CO_2 hydration in the presence of choline chloride salts. Open circles 0.0076 M CO_2: Y = 1.61 X - 1.86; Shaded circles 0.0228 M CO_2: Y = 1.74 X - 6.97.

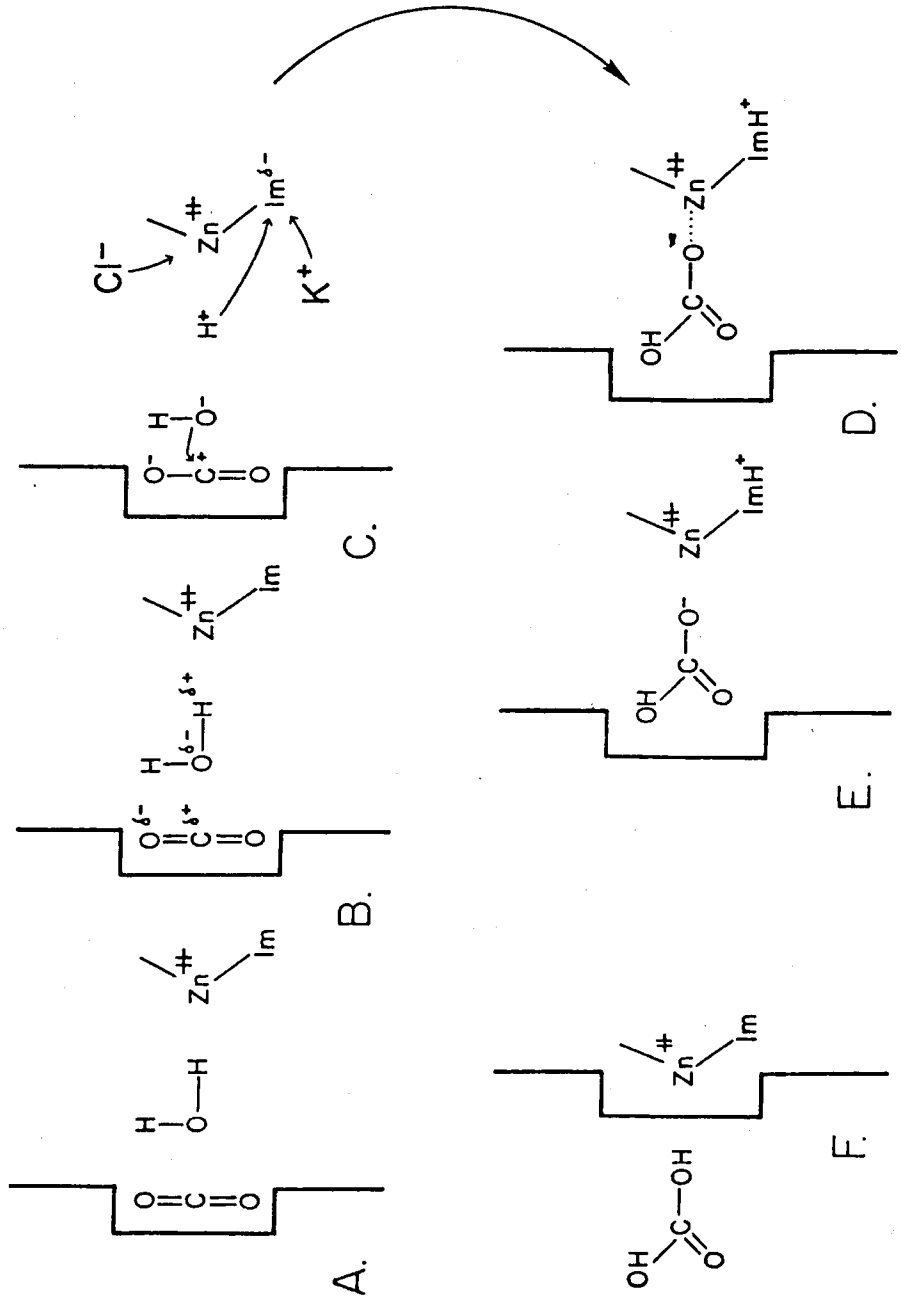


Figure 6.5. Schematic representation of the events at the catalytic site of carbonic anhydrase during CO_2 hydration (A ⟶ F) of HCO_3^- dehydration (F ⟶ A). The effects of cations or anions during enzyme catalysis is diagramatically represented in C.

halide displacement during catalysis would be competitive. During carbonic anhydrase catalysed bicarbonate (HCO_3^-) dehydration, halides would compete with bicarbonate for access to the catalytic site. Here also halide anions would kinetically compete competitively with HCO_3^-.

Considerable controversy exists regarding the kinetics of halide anion inhibition during C.A. catalysed CO_2 hydration. Workers in two different laboratories have found that halide anions compete noncompetitively during C.A. catalysed CO_2 hydration and competitively during C.A. catalysed HCO_3^- dehydration (Maren, Rayburn and Liddell, 1976; Pocker and Tanaka, 1978). This conclusion has been questioned by Koenig and Brown (1976), who argue that the effects of halide anions must be the same for the forward (CO_2 hydration) and reverse directions (HCO_3^- dehydration). In this paper, we have determined that chloride anions when present as the choline salt competitively inhibit C.A. catalysed CO_2 hydration (Figure 6.3), but when present as the potassium salt <u>noncompetitively</u> inhibit CO_2 hydration. The controversy regarding halide anion inhibition during Bovine or Human B catalysed CO_2 hydration is now resolved, since Maren, Rayburn and Liddell (1976) and Pocker and Tanaka (1978) both employed potassium (or sodium) halide salts in their respective studies.

The nature of the K^+ effect in mammalian C.A.'s during CO_2 hydration, and the stimulatory effect of K^+ on the insect enzymes can now be resolved. One difference between C.A. catalysed CO_2 hydration and HCO_3^- dehydration is the role of the histidyl imidazole group. Bicarbonate dehydration is catalysed preferentially at high H^+ ion

concentrations (acidic pH). Since K^+ does not appear to influence halide anion competitive inhibition of mammalian C.A. catalysed HCO_3^- dehydration, but it does influence C.A. catalysed CO_2 hydration, one can then predict that K^+ (Or other monovalent alkali metal cations) noncompetitively competes with H^+ for the histidyl imidazole group. This competition between K^+ and H^+ is so extensive as to dominate the kinetics of catalysis. At alkaline pH's, where CO_2 hydration is favored, the concentration of H^+ available to the histidyl imidazole group is reduced to a level such that at concentrations as low as 5-10 mM, K^+ effectively displace H^+ (Figure 6.5C). At acid pH's, K^+ unsuccessfully competes with H^+ and competition between halide anion and bicarbonate will determine the kinetics of catalysis.

Analysis of cation and anion effects on insect C.A. are more perplexing. Two possible explanations for the stimulatory effects of K^+ (in contrast to the inhibition exhibited by mammalian enzymes, Table 6.1, p. 137) are: the catalytic site and mechanism of catalysis by insect enzymes differs drastically from its mammalian counterpart, or insect enzymes are positively affected allosterically by alkali metal cations. The former possibility appears unlikely, since insect C.A.'s are inhibited by diamox sulfate (Johnston and Jungreis, 1978), and the I_{50}'s (concentration that will cause a 50% reduction in catalytic activity) for the insect enzymes are comparable to that observed for bovine C.A. (Coleman, 1967; Johnston and Jungreis, in preparation). The alternate model that K^+ allosterically affects insect C.A. is not entirely unreasonable, since K^+ is the principal alkali metal cation in

hemolymph of M. sexta (Jungreis, Jatlow and Wyatt, 1973; Jungreis, 1978). Further, since the principle anions present in hemolymph are organic in composition (Jungreis, 1978; Jungreis and Omilianowski, in preparation), an increased entry of K^+ across the midgut epithelium via coupled exchange with H^+ into hemolymph would necessitate an increase in bicarbonate production to both supply H^+ needed in exchange diffusion with K^+, and for electroneutrality (via HCO_3^-) of the incoming K^+. In all events, the effects of K^+ on insect C.A. appears to be fundamentally different from its effect on mammalian C.A.

CHAPTER 7

ION TRANSPORT BY INTEGUMENTARY EPITHELIUM OF THE TOBACCO HORNWORM (Manduca sexta) DURING SECRETION AND RESORPTION OF LARVAL-PUPAL MOULTING FLUID

I. INTRODUCTION

Fluid and ion movements have been studied in a wide variety of insect systems. The midgut (see Harvey and Zerahn, 1972; Blankemeyer and Harvey, 1978, for a summary of the history of the midgut system in Hyalophora cecropia and Manduca sexta), salivary (Berridge, et al., 1976) and labial glands (Kafatos, 1968), rectum (Phillips, et al., 1977; Goh and Phillips, 1978; Williams, et al., 1978) and malpighian tubules (Berridge, 1969; Maddrell, 1969, 1971) have been extensively studied in terms of water and ion movement. However, little attention has been paid to that system involved in fluid and ion movement during secretion and resorption of moulting fluid by the integument (see review by Jungreis, 1979).

The physiology of moulting has been studied from a number of approaches, including hormonal control, protein and nucleic acid metabolism, cuticular morphology and selective degradation of the "old" cuticle (see Jungreis, 1979, for an extensive review), yet little or no attention has been paid to the moult process proper. Basic questions dealing with the driving forces behind moulting fluid secretion and resorption have not been resolved. Furthermore, the absence of

generalized models on the origin of the electrochemical gradients measured during ecdysis have made interpretation of experimental results more difficult. In this paper, I provide insight into which ions are actively or passively transported, when and why across the larval-pupal integument of Manduca sexta. A model is proposed wherein K^+ and H^+, actively transported during secretion of moulting fluid, are responsible for fluid movements from hemolymph to exuvial side, while active transport of bicarbonate is responsible for fluid movements from exuvial to hemolymph sides of the integument during resorption of moulting fluid.

II. MATERIALS AND METHODS

General Conditions

Tobacco hornworm used in these experiments were obtained from an inbred colony maintained in our laboratory. Larvae were reared on the wheat germ-casein diet of Bell and Joachim (1976) under an 12L:12D photoperiod regimen at 24 to 25°C. Animals were staged according to the criterion of Jungreis (1978).

Tissue Mounting Procedures

The pharate pupal integument is dissected from the larval cuticle at 2 to 3 hours before ecdysis, when it consists of a delicate cuticle over a one cell thick layer of pupal integumentary epithelial cells. Midgut, fat body and adhering tissues are gently scraped from the hemolymph side of the integument and the larval cuticle removed by inserting blunt-tipped forceps between the two layers and separating

them carefully at the major points of attachment, the spiracular junctions. The isolated integument is transferred as a flat sheet to a plexiglass chamber (see Wood, 1972) and tied in place with cotton twine. An area of 1.0 cm^2 is then exposed to identical but isolated bathing solutions. The standard solution consists of 32 mM $KHCO_3$, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 15 mM Tris-HCl (pH 8.6), and 180 mM Sucrose, and a total osmotic equivalent of about 270 mOsms. The bathing solutions were continuously stirred and oxygenated by bubbling 100% medical grade O_2 into each side of the chamber. All experiments were carried out at 23°C.

The pharate pupal integument was short-circuited as a flat sheet by the Wood modification (1972) of the classical techniques of Ussing and Zerahn (1951). The short circuiting, as well as the determination of potential difference, was accomplished by using an automatic voltage-clamping device designed by Dr. James T. Blankemeyer, Oklahoma State University. This device passes current through the solutions via Ag-AgCl plates such that the tissue can be clamped at any desired potential. A three bridge system is employed to correct for voltage drop through the bathing solution due to the solution resistance. The summated pump resistances of the integumentary epithelium are determined by applying a 50 microAmp cm^{-2} square pulse of current briefly across the integument and recording the trans-epithelial potential difference. Resistances were calculated from the current and the measured potential difference using Ohm's law.

Flux Measurements

The normal procedure employed in studying trans-epithelial ion fluxes is via addition of an isotopic marker to one side of the tissue and then sampling the "cold" side over an extended period of time. Following this, both sides are rinsed, the isotopic marker added to the previously cold side and movement in the opposite direction monitored.

The integument of M. sexta has an extremely large exchangeable intracellular pool. Following measurement of the isotopic flux in the initial direction, this intracellular pool washes out into the chamber at a rate greatly in excess of the flux in the final direction. This wash out occurs not only during rinsing procedures (Figure 7.1), but also when N_2 replaces O_2 as the bathing gas (Figure 7.2). This intracellular washout is so large that 7 hours after multiply rinsing the tissue, the flux due solely to washout is twice that of the flux initially measured, while as much as 25 hours post rinse the washout flux is equal to the initial flux (Figure 7.1). This washout is also seen when the supply of O_2 to the chamber is replaced with N_2. Under these conditions the I_{sc} drops rapidly (about 20 minutes) to near zero, while the measured flux more than doubles within an hour (Figure 7.2). In light of these observations, it is impossible to accurately study ionic fluxes across the integument of M. sexta by the usual procedures, namely, studying flux from (e.g.) hemolymph to exuvial side, wash out the "hot side" and measure the flux from the exuvial to hemolymph side. This washout problem can be circumbented by studying unidirectional fluxes on separate tissues, a technique I refer to as "half-cell

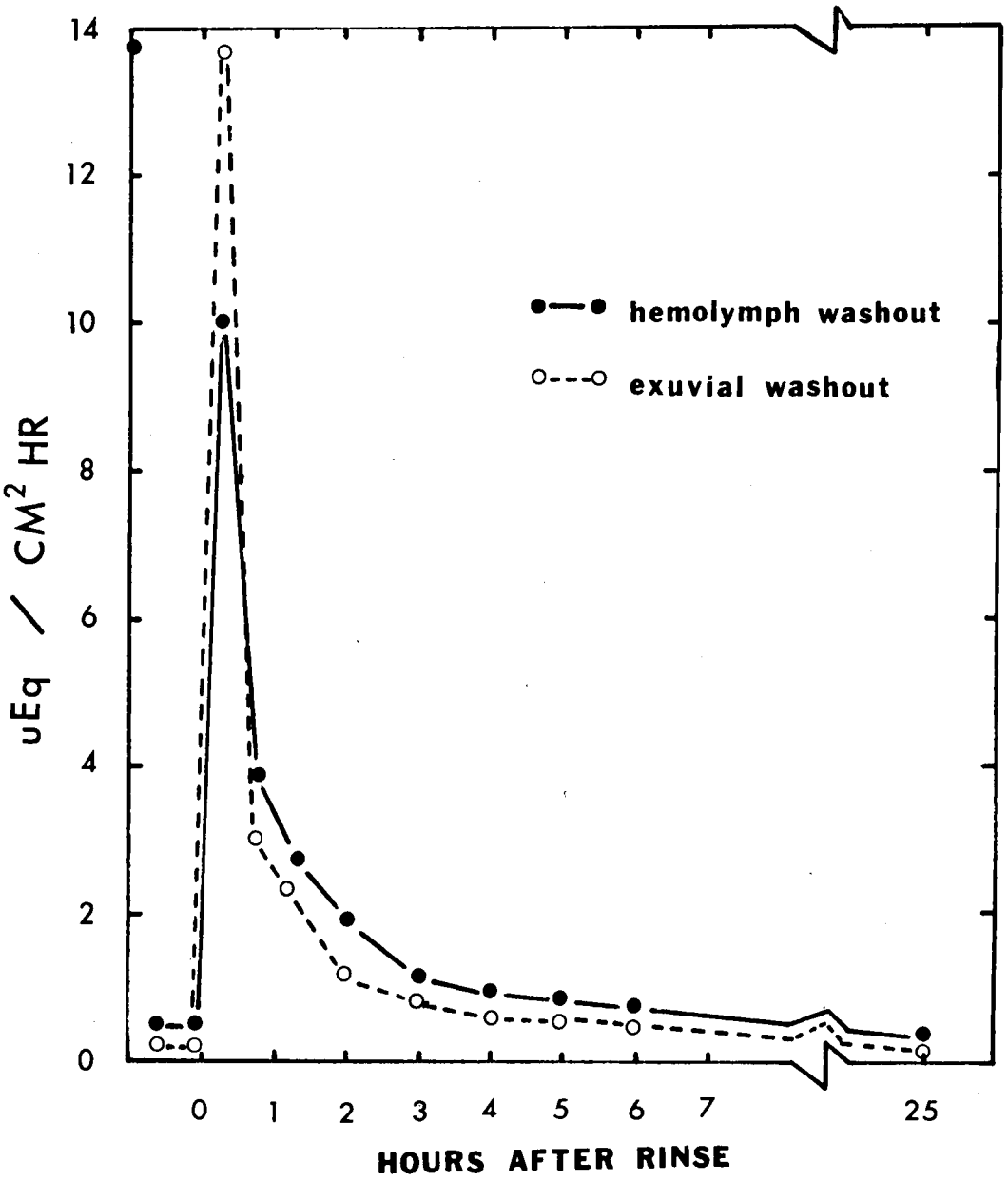


Figure 7.1. Typical washout rates seen following multiple rinses of both sides of the integument. Closed circles-solid line represents isotope washout onto the hemolymph side; open circles-dashed line represents isotope washout onto the exuvial side. Isotope used in this study was ^{86}Rb; the tissue was 15-12 hours before ecdysis.

Figure 7.2. ^{86}Rb fluxes following replacement of O_2 with N_2. In the left hand figure the hemolymph side was labeled so that the washout flux was toward the exuvial side. In the right hand figure the exuvial side was labeled so that the washout flux was toward the hemolymph side. The arrows marked N_2 indicate the point at which O_2 was removed as the bubbling gas and N_2 substituted.

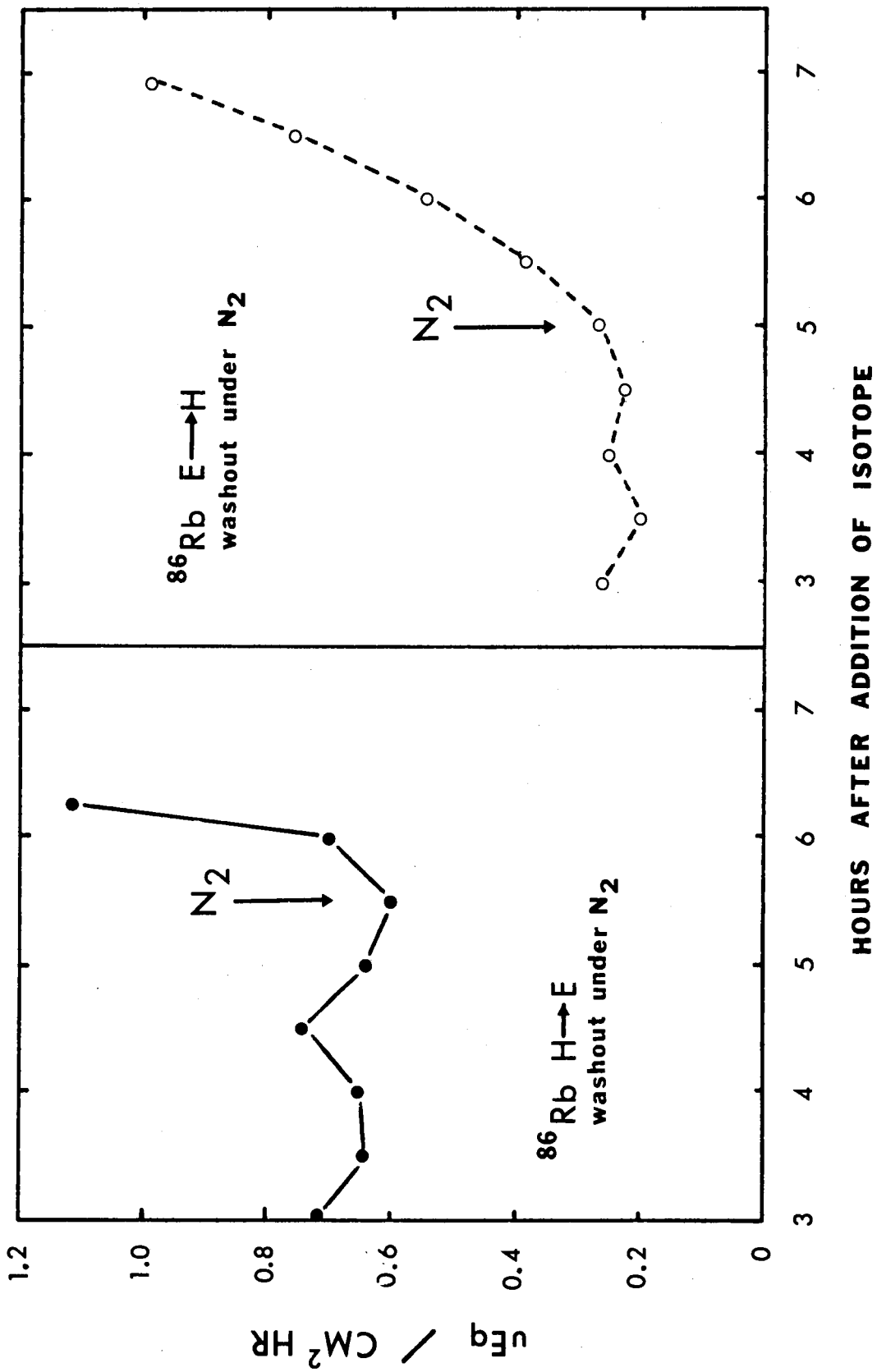

1.2
1.0
0.8
0.6
0.4
0.2
0
uEq / CM² HR
N2
86Rb H→E
washout under N2
N2
86Rb E→H
washout under N2
3
4
5
6
7
HOURS AFTER ADDITION OF ISOTOPE

Figure 7.2

preparation." The validity of this approach was established by demonstrating that the net flux of potassium across the integument is the same when half-cell preparations are used, or when the influx and efflux are measured on the same tissue using ^{42}K and ^{86}Rb as markers for potassium transport (Table 7.1). In this latter approach, which I have designated "whole-cell preparation," ^{42}K is added to one side of the tissue and the unidirectional flux monitored after a 2 hour equilibration by immediately counting samples taken from the cold side. Following this procedure both sides of the chamber were rinsed and ^{86}Rb added to the previously "cold" side. Samples taken from the "cold" side (previously the "hot" side) were counted one week later (i.e., after ^{42}K had gone through about 24 half lives). By using ^{42}K in the initial direction and ^{86}Rb in the final direction, any ^{42}K which washes out during the study of ^{86}Rb in the final direction will have decayed to zero activity, and would no longer interfere with measurements in the final direction.

I found no significant difference in either net fluxes or percentages of the short-circuit current represented by the stage specific flux between the whole-cell preparations (Table 7.1) and half-cell preparations (Table 7.2). The direct comparison of unidirectional fluxes on separate tissues at the same stage is therefore valid.

Unidirectional fluxes of $^{86}Rb^{+}$, $(^{14}C)\text{-}HCO_3^{-}$, $^{36}Cl^{-}$ and $^{45}Ca^{++}$ were measured under short-circuit and open-circuit conditions. When addition of isotope to one side of the membrane changed ion concentrations of the bathing media significantly (e.g., $^{36}Cl^{-}$ experiments), an unlabeled but

TABLE 7.1. ^{42}K and ^{86}Rb fluxes during the larval-pupal transformation in M. sexta. All determinations were made via the whole-cell preparation (see Methods section). Eighteen to 12 hours represents secretion while 9 to 3 hours before ecdysis represents resorption. The 12 to 9 hour segment is transitional between secretion and resorption.

	Hours Before Ecdysis				
	18-15	15-12	12-9	9-6	6-3
Flux H ⟶ E ($uEq\ cm^{-2}hr^{-1}$)	.343±.029 n=3	.549±.061 n=3	.279±.027 n=4	.194±.021 n=3	.051±.006 n=3
Flux E ⟶ H ($uEq\ cm^{-2}hr^{-1}$)	.134±.015 n=3	.234±.031 n=3	.196±.018 n=4	.240±.026 n=3	.104±.007 n=3
Net Flux ($uEq\ cm^{-2}hr^{-1}$)	.209±.021 n=3	.315±.045 n=3	.083±.003 n=4	.046±.004 n=3	.053±.004 n=3
Jet Direction	H ⟶ E	H ⟶ E	H ⟶ E	E ⟶ H	E ⟶ H
Average I_{sc} ($u\ Amps\ cm^{-2}hr^{-1}$)	15.5	12.2	13.5	15.4	8.62
Percentage Agreement	+ 36.1	+ 69.0	+ 16.4	- 8.0	- 16.5

n = the number of individual determinations; numbers are means ± the standard error of the mean.

TABLE 7.2. ^{86}Rb fluxes throughout moulting fluid secretion (21 to 12 hours before ecdysis) and resorption (9 to 3 hours before ecdysis). The 12 to 9 hour period represents a transitional period. All measurements were made via the half-cell method (see Methods).

	Hours Before Ecdysis					
	21-18	18-15	15-12	12-9	9-6	6-3
Flux H ⟶ E ($uEq\ cm^{-2}hr^{-1}$)	.392±.041 n=4	.387±.029 n=7	.510±.021 n=3	.202±.016 n=3	.178±.019 n=3	.074±.006 n=6
Flux E ⟶ H ($uEq\ cm^{-2}hr^{-1}$)	.179±.008 n=4	.160±.006 n=4	.181±.009 n=3	.119±.004 n=3	.229±.036 n=4	.084±.009 n=5
Net Flux ($uEq\ cm^{-2}hr^{-1}$)	.213	.227	.329	.083	.051	.010
Net Direction	H ⟶ E	H ⟶ E	H ⟶ E	H ⟶ E	E ⟶ H	E ⟶ H
Average I_{sc} ($u\ Amps\ cm^{-2}hr^{-1}$	16.95	17.55	15.60	14.95	19.70	4.77
Percentage Agreement	+ 33.7	+ 34.6	+ 56.5	+ 15.0	- 6.9	- 5.6

n = the number of individual determinations.

± = the standard of the mean.

otherwise equivalent solution was added in the same amount on the opposite side so that electrochemical or pH gradients were not established across the integumentary epithelium. Several hours after isotope is added to the "hot" side, a 100 microliter aliquot is removed to determine the specific activity on this side. Following a minimum of at least two hours for equilibration, an aliquot (2 ml) is removed from the "cold" side every 30 minutes to determine the quantity of material which has crossed the integument. The fluid removed is then replaced with the same volume of unlabeled saline. Under these conditions it is possible to estimate the unidirectional fluxes over the 30 minute interval using the following equation:

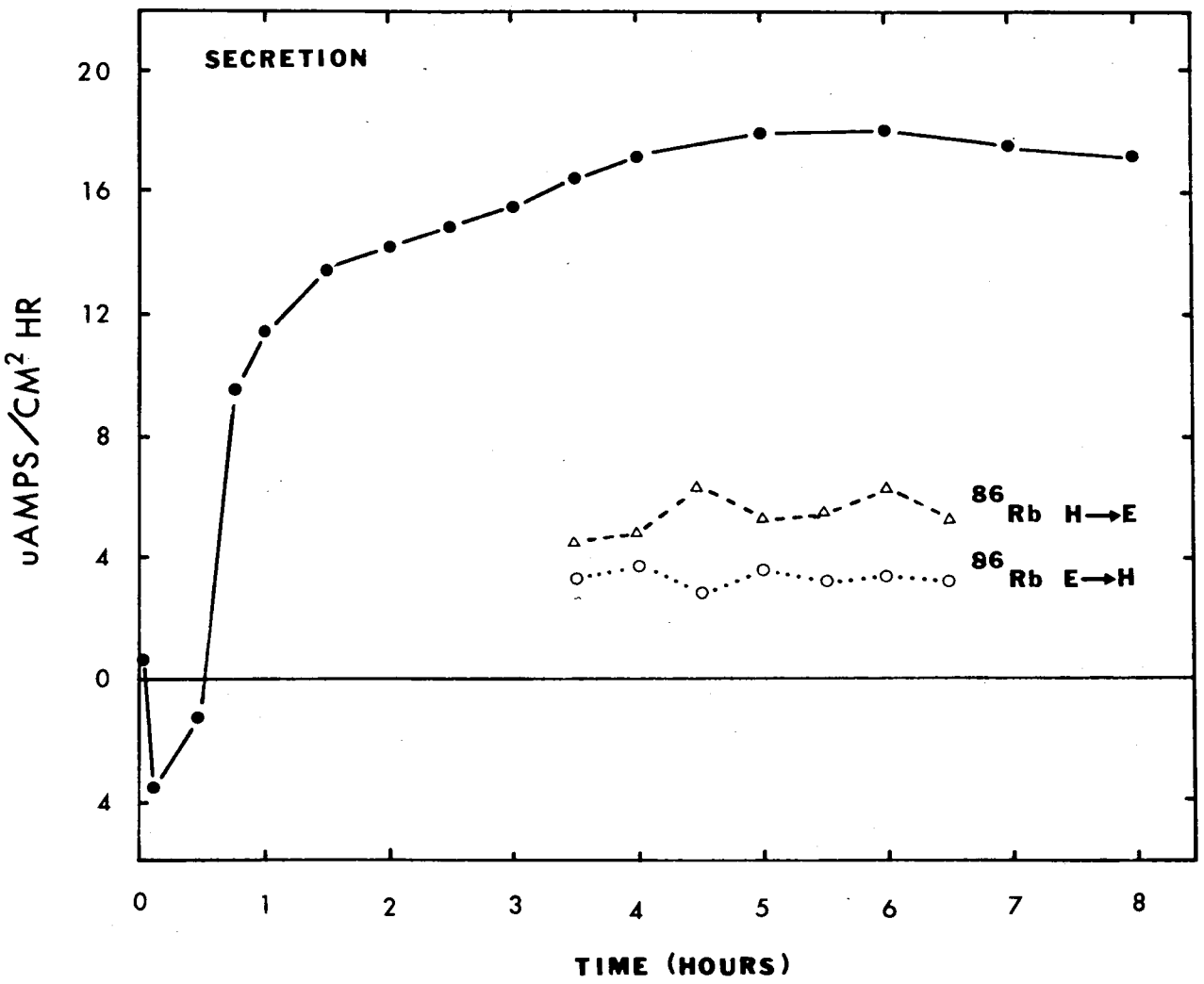
$$J_{1 \longrightarrow 2} = \frac{a_2 \times V \times C}{a_1 \times T \times A}$$

where $J_{1 \longrightarrow 2}$ is the unidirectional flux in uEq $cm^{-2}hr^{-1}$, a_1 represents the radioisotopic activity (counts $min^{-1}ml^{-1}$) on side 1, a_2 is the increase in radioactivity on side 2 (counts $min^{-1}ml^{-1}$) over the 30 minute interval, V is the volume of fluid on side 2 in mls (13.5 to 17.1 depending on the chamber), C is the concentration of unlabeled ion in the saline solution (uEq ml^{-1}), T is the time interval over which the flux occurred (0.50 hours), and A is the area of the tissue (1.0 cm^2).

III. RESULTS

Viability of the Preparation

The long term viability of the *in vitro* preparation, as indicated by the short-circuit current, is shown in Figure 7.3. The I_{sc} during



igure 7.3. Typical short-circuit current decay profile seen during secretion of moulting The typical unidirectional fluxes of ^{86}Rb during this period are also shown.

secretion is initially low and often in the wrong direction, but rises rapidly to a value usually between 10 to 25 uAmps cm^{-2} hr^{-1} within 1 hour. Thereafter the I_{sc} is relatively constant for as long as 10 to 12 hours. During late resorption (6-3 hours before ecdysis) the magnitude of the current is significantly lower (1 to 8 uAmps cm^{-2} hr^{-1}), decays much more rapidly (Figure 7.4), but with a steady state of at least 6 to 8 hours. During the steady state phase the potential difference (PD, range during secretion is 10 to 15 mV, exuvial side positive with respect to hemolymph; range during resorption is usually 1 to 6 mV exuvial side positive with respect to hemolymph) and resistance (range 500 to 1000 Ωcm^2) are invarient.

IV. SOURCE OF THE I_{sc}: RADIOISOTOPE FLUX STUDIES

During periods of moulting fluid secretion (21 to 9 hours before ecdysis) the net flux of K^+ from hemolymph to exuvial space (i.e., moulting fluid) accounts for only 34 to 70% of the I_{sc} (Tables 7.1 and 7.2). During periods of moulting fluid resorption (9 to 3 hours before ecdysis) net K^+ flux is in the opposite direction (exuvial space to hemolymph) to that of the I_{sc} and is therefore in disagreement (negative percentage) with that current.

The apparent net flux of HCO_3^- is always in the direction from hemolymph to exuvial side of the integument (Table 7.3). The unidirectional flux of HCO_3^- from exuvial to hemolymph side of the integument is high throughout development (.238 uEqs $cm^{-2}hr^{-1}$ at 15 to 12 hours before ecdysis versus .214 uEqs $cm^{-2}hr^{-1}$ at 9-6 hours before ecdysis). The

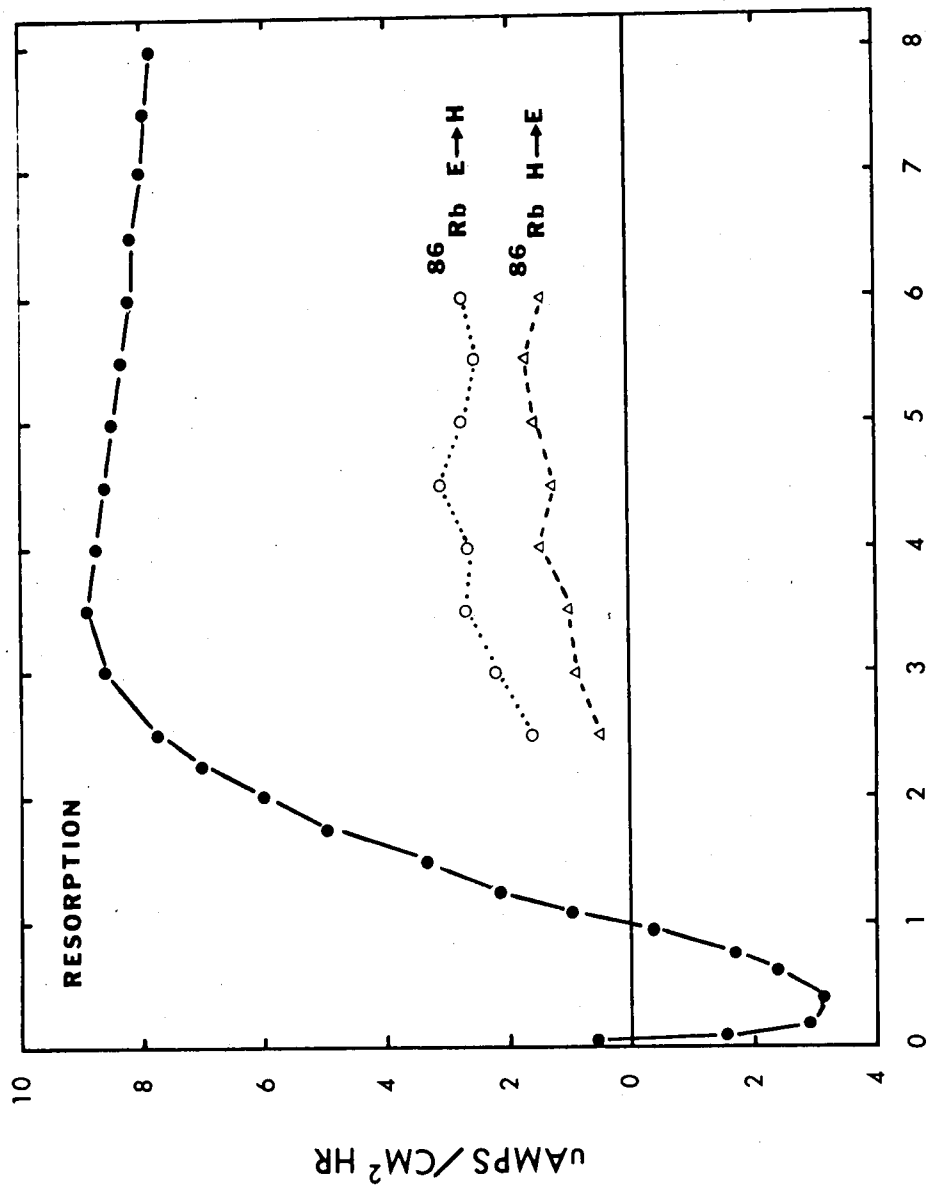


Figure 7.4. Typical short-circuit current decay profile seen during resorption of larval-pupal moulting fluid across the integument of M. sexta. Typical unidirectional ^{86}Rb fluxes for this period are also included.

TABLE 7.3. (^{14}C)-HCO_3^- fluxes during the larval-pupal transformation in M. sexta. See legend for Table 7.2.

	Hours Before Ecdysis					
	21-18	18-15	15-12	12-9	9-6	6-3
Flux H ⟶ E ($uEq\ cm^{-2}hr^{-1}$)	.556±.061 n=4	.485±.039 n=5	.660±.058 n=3	.462±.037 n=3	.292±.016 n=3	.340±.021 n=5
Flux E ⟶ H ($uEq\ cm^{-2}hr^{-1}$)	.147±.009 n=4	.158±.011 n=5	.162±.009 n=3	.238±.019 n=5	.214±.015 n=3	.169±.007 n=4
Net Flux ($uEq\ em^{-2}hr^{-1}$)	.409	.327	.498	.224	.078	.171
Net Direction	H ⟶ E	H ⟶ E	H ⟶ E	H ⟶ E	H ⟶ E	H ⟶ E
Average I_{sc} ($uAmps\ cm^{-2}hr^{-1}$)	15.3	17.5	15.0	16.2	19.6	4.8
Percentage Agreement	- 71.6	- 50.1	- 88.9	- 37.0	- 10.7	- 95.9

n = the number of individual determinations.

± = the standard of the mean.

tendency for HCO_3^- to move from hemolymph to exyvial side opposes the development of a positive potential across the integument (exuvial space positive relative to hemolymph). For this reason agreement between net flux of HCO_3^- and the measured I_{sc} is expressed as a negative number.

During periods of moulting fluid secretion there is no significant net flux of Cl^- in either direction (Table 7.4). During resorption of moulting fluid there is a net exuvial to hemolymph flux of Cl^- which accounts for 12 to 15% of the I_{sc}.

The unidirectional fluxes of $^{45}Ca^{++}$ are identical during both resorption and secretion (.0072 uEqs $cm^{-2}hr^{-1}$ hemolymph to exuvial and .0056 uEqs $cm^{-2}hr^{-1}$ exuvial to hemolymph). The net flux of Ca^{++} is extremely small and accounts for a negligible portion of the I_{sc} (Table 7.5), but its presence in the bathing medium is absolutely necessary for the maintenance of the I_{sc} (see Jungreis, 1979). The magnitude of the Ca^{++} fluxes indicates that there are no leaks nor tissue edge damage. Neither the short-circuit current nor the unidirectional fluxes of K^+, HCO_3^-, Cl^- or Ca^{++} were significantly different in the absence or presence of Mg^{++} (data not shown), nor were unidirectional fluxes under short-circuit conditions significantly different from those measured under open-circuit conditions (data not shown).

Data for the net fluxes of K^+, HCO_3^-, Cl^- and Ca^{++} during secretion (data for 21 to 12 hours before ecdysis have been averaged) and resorption (data for 9 to 3 hours before ecdysis have been averaged) of moulting fluid are summarized in Table 7.5. The 12 to 9 hour period before ecdysis is a transitional period between secretion and resorption

TABLE 7.4. ^{36}Cl fluxes during the larval-pupal transformation in M. sexta. See the legend for Table 7.2.

	Hours Before Ecdysis					
	21-18	18-15	15-12	12-9	9-6	6-3
Flux H ⟶ E ($uEq\ cm^{-2}hr^{-1}$)	.238±.022 n=3	.106±.021 n=3	.109±.011 n=3	.039±.005 n=3	.082±.009 n=3	.034±.004 n=3
Flux E ⟶ H ($uEq\ cm^{-2}hr^{-1}$)	.240±.021 n=3	.105±.009 n=3	.111±.010 n=3	.110±.009 n=3	.189±.012 n=3	.058±.009 n=5
Net Flux ($uEq\ cm^{-2}hr^{-1}$)	.002	.001	.002	.071	.107	.024
Net Direction	E ⟶ H	H ⟶ E	E ⟶ H	E ⟶ H	E ⟶ H	E ⟶ H
Average I_{sc} ($Amps\ cm^{-2}hr^{-1}$	15.3	17.6	15.6	16.2	19.7	4.8
Percentage Agreement	+ .35	- .15	+ .34	+ 11.8	+ 14.6	+ 13.5

n = the number of individual determinations.

± = the standard of the mean.

TABLE 7.5. Summary of the net fluxes of K^+, HCO_3^-, Cl^- and Ca^{++} during secretion and resorption of moulting fluid in the larval-pupal transformation of M. sexta. The averages for K^+ fluxes are from Tables 7.1 and 7.2. HCO_3^- fluxes are from Table 7.3 and Cl^- movements from Table 7.4. See Results section for unidirectional Ca^{++} fluxes.

Ion	Average Net Flux During Secretion (21 to 12 hours before ecdysis)	Direction	% of I_{sc}	Average Net Flux During Resorption (9 to 3 hours before ecdysis)	Direction	% of I_{sc}
K^+	.259 uEq $cm^{-2}hr^{-1}$	H ⟶ E	+ 46	.040 uEq $cm^{-2}hr^{-1}$	E ⟶ H	- 9
HCO_3^-	.411 uEq $cm^{-2}hr^{-1}$	H ⟶ E	- 70	.125 uEq $cm^{-2}hr^{-1}$	H ⟶ E	- 53
Cl^-	.001 uEq $cm^{-2}hr^{-1}$	H ⟶ E	+ .18	.066 uEq $cm^{-2}hr^{-1}$	E ⟶ H	+ 12
Ca^{++}	.002 uEq $cm^{-2}hr^{-1}$	H ⟶ E	+ .35	.002 uEq $cm^{-2}hr^{-1}$	H ⟶ E	+ .85

and has therefore been omitted. The two most obvious points seen from Table 7.5 are:

1. There must be another transported species along with K^+ in order to account for the I_{sc}. This could be either a cation transported hemolymph to exuvial or an anion transported exuvial to hemolymph.
2. In the face of an electropositive exuvial space (with respect to hemolymph) the transport of HCO_3^- cannot be electrogenically coupled to K^+ transport.

V. DISCUSSION

Active transport of K^+ across the integumentary epithelium of H. cecropia (hemolymph to exuvial) accounts for 100% of the I_{sc} during secretion of moulting fluid, but only 10% during resorption of moulting fluid (Jungreis and Harvey, 1975). In M. sexta active transport of K^+ can account for only half the I_{sc} during secretion of moulting fluid and none during the resorption of moulting fluid (Table 7.5). In a recent study on the composition of hemolymph and moulting fluid in M. sexta (Chapter 2) the pH of moulting fluid was noted to be approximately 7.2 during all phases of secretion and resorption. However, based on the Henderson-Hasselbalch relationship, the 100 mM concentration of bicarbonate (Chapter 2) should be associated with a pH of 8.5 rather than the observed pH of 7.2. As an explanation for the presence of a higher H^+ ion concentration (lower pH) in moulting fluid, I propose that H^+ derived from carbonic acid competes with K^+ for sites on the K^+ pump

and is actively transported through the K^+ pump into the exuvial space during secretion of moulting fluid. The movement of approximately one H^+ ion for every K^+ would account for the missing 50% of the I_{sc}. When HCO_3^- is replaced by Cl^- in the bathing medium, the I_{sc} falls by approximately 50% within 15 minutes and is maintained at this lower level indefinitely. When HCO_3^- is added back to the bathing solution the I_{sc} immediately doubles. Furthermore, if Tris Buffer is omitted from the bathing solution the pH of the exuvial space becomes increasingly more acidic. Thus by analogy with the situation in vivo, the missing component of the I_{sc} is probably a cation (namely, H^+) moving hemolymph to exuvial rather than an anion moving exuvial to hemolymph.

Net bicarbonate movement is always in the direction H $\longrightarrow$ E at all phases of moulting fluid elaboration. Since the magnitude of this movement is so large (63% higher than K^+), and if HCO_3^- and K^+ movements were electrically coupled, then the exuvial space would be electronegative with respect to the hemolymph. Since the exuvial space is always electropositive, both in vivo and in vitro (see Jungreis, 1979), the movement of HCO_3^- cannot be electrogenically coupled to the movement of K^+ and therefore contributes little to the observed P.D. during secretion of moulting fluid. However, during resorption of moulting fluid, electrogenic transport of HCO_3^- must occur (see below). The major roles of the nonelectrogenic bicarbonate pump are:

1. to buffer the moulting fluid to a pH favorable for hydrolytic enzymes in the exuvial space,

2. to provide osmotic equivalents to aid in the production of an iso-osmotic moulting fluid (with respect to hemolymph, see Jungreis, 1979), and

3. to aid in providing electroneutrality of the moulting fluid.

I envision the nonelectrogenic transport of HCO_3^- as occurring across the lateral spaces between the integumentary epithelial cells with its subsequent "coupled" movement with K^+ across the apical border into the exuvial space (see Figure 7.5).

The driving force behind resorption of the moulting fluid is difficult to discern in light of the absence of net ion fluxes in the direction E $\longrightarrow$ H (Table 7.5). That fluid is indeed being resorbed in vitro is evidenced by a reversal in the direction of the net flux of K^+ (Tables 7.1 and 7.2, p. 157-158). During resorption, I propose that HCO_3^- is transported by two separate pumps, a nonelectrogenic lateral surface pump and an electrogenic basal surface pump (Figure 7.5). It is obvious that the net movement of HCO_3^- (H $\longrightarrow$ E) during secretion of moulting fluid continues or appears to continue during resorption of moulting fluid, although to a much smaller degree since net fluxes are reduced from 65 to 24% of the I_{sc} (Table 7.3, p. 163). I propose that this reduction is due to the "activation" of a second bicarbonate pump situated on the basal surface of the epidermal cells, and that movement of HCO_3^- (E $\longrightarrow$ H) through this pump drives the resorption of moulting fluid (Figure 7.5). Some of the HCO_3^- equivalents available for transport through the second, electrogenic HCO_3^- pump could arise from metabolically produced CO_2, so that net flux measurements is the presence

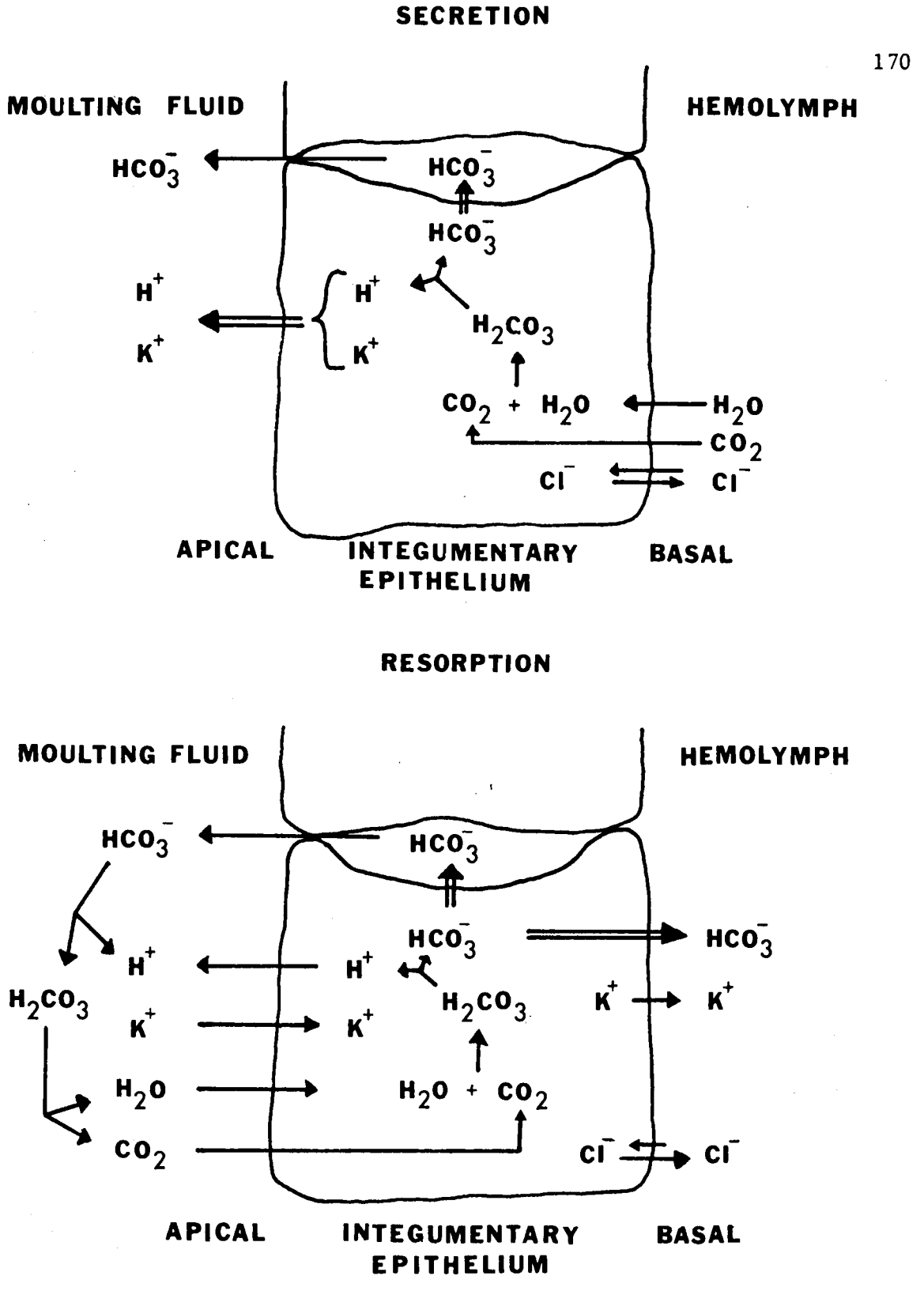


Figure 7.5. Proposed model for ion and water movements during secretion (top figure) and resorption (bottom figure) of larval-pupal moulting fluid across the integumentary epithelium of M. sexta.

of (^{14}C)-HCO_3^- may not represent the entire HCO_3^- pool. If this is in fact the case, then the flux of HCO_3^- (E $\longrightarrow$ H) during moulting fluid resorption could be substantially larger.

During secretion of moulting fluid a net flux of Cl^- is not observed (Table 7.4, p. 165). However, during resorption the net flux of Cl^- accounts for about 15% of the I_{sc} (Table 7.4). I believe that this net flux is primarily an artifact of the in vitro preparation. The major factor behind the increased net flux E $\longrightarrow$ H is a decreased membrane permeability towards Cl^- in the direction H $\longrightarrow$ E. This reduction in permeability probably reflects increased synthesis of the endocuticle. However, even if the Cl^- flux is real, it could account for only 15% of the observed I_{sc}.

A model is proposed wherein the coupled movement of K^+ and H^+ accounts for the entire I_{sc} during secretion of moulting fluid (Figure 7.5). The K^+ pump is located on the apical surface of the epidermal cells as indicated by pH and O_2 sensitivity on the apical (= exuvial) surface only (Jungreis, 1979). Bicarbonate, produced by intercellular carbonic anhydrase and probably metabolically produced CO_2, is pumped into the lateral space between epithelial cells. Protons produced along with HCO_3^- are then transported through the K-pump onto the exuvial side. Viewed under the light microscope, spidermal cells do exhibit an extensive intracellular space during moulting fluid secretion and resorption. This mechanism accounts for:

1. production of high levels of K^+ and HCO_3^- in moulting fluid (see Chapter 2),

2. the maintenance of only a slightly alkaline pH in spite of high levels of HCO_3^- in moulting fluid,
3. the nonelectrogenic movement of HCO_3^-, and
4. the driving force for H_2O movement down from an electrochemical gradient established by movement of K^+, H^+ and HCO_3^- into moulting fluid (see Figure 7.5).

During resorption of moulting fluid, I propose that an electrogenic HCO_3^- pump is established on the basal surface of the epidermal cells, which transports HCO_3^- in the direction E $\longrightarrow$ H. The magnitude of this electrogenic pump is smaller than the nonelectrogenic pump moving HCO_3^- into the lateral space, but would still allow for a driving force for moulting fluid resorption (Table 7.3, p. 163, and Figure 7.5). HCO_3^- equivalents formed from metabolically produced CO_2 may enhance the magnitude of this electrogenic pump. During resorption, K^+ is no longer actively transported H $\longrightarrow$ E, but rather has a net flux E $\longrightarrow$ H. I propose that K^+ passively exchanges for a proton across the apical surface of the integumentary epidermal cell, and then accompanies the HCO_3^- being moved by the basal bicarbonate pump (Figure 7.5). The H^+ ion entering moulting fluid in exchange for K^+ combines with HCO_3^- in moulting fluid as a vehicle to recycle CO_2 and H_2O (see Figure 7.5). This mechanism would account for the invarient pH (7.2) and osmotic pressure (ca. 300 mOsms) of moulting fluid (Jungreis, 1978) and would provide for the driving force behind moulting fluid resorption.

REFERENCES

REFERENCES

Anderson, A. D., and March, R. B. (1956). Inhibitors of carbonic anhydrase in the American cockroach, Periplaneta americana (L.). Can. J. Zool. 34:68-74.

Bade, M. L. (1978). Enzymatic breakdown of the chitin component in insect cuticle during the molt. Personal Communication.

Bade, M. L. (1975). The pattern of appearance and disappearance of active molting chitinase in Manduca cuticle. The endogneous activity. F.E.B.S. Letters 51:161-163.

Bade, M. L. (1974). Localization of milting chitinase in insect cuticle. Biochem. Biophys. Acta. 372:474-477.

Bade, M. L., and Shoukimas, J. (1974). Neutral metal chelator-sensitive protease in insect molting fluid. J. Insect Physiol. 20:281-290.

Berridge, M. J. (1969). Urine formation by the malpighian tubules of Calliphora, II: Anions. J. Exp. Biol. 50:15-28.

Berridge, M. J., Lindley, B. D., and Prince, W. T. (1976). Studies on the mechanism of fluid secretion by isolated salivary glands of Calliphora. J. Exp. Biol. 64 (2):311-322.

Bell, R. A., and Joachim, F. G. (1976). Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. Ann. Ent. Soc. Am. 69:363-373.

Bishop, G. H. (1953). Body fluid of the honeybee larva I. Osmotic pressure, specific gravity, pH, O_2 capacity, CO_2 capacity and burrer value, and their changes with larval activity in metamorphosis. J. Biol. Chem. 58:543-565.

Blankemeyer, J. T., and Harvey, W. R. (1978). Identification of active cell in potassium transporting epithelium. J. Exp. Biol. 77:1-13.

Boyce, P. K. W. (1978). Regulation of fat body urate accumulation in the development of the tobacco hornworm, Manduca sexta. M.S. Thesis, University of Tennessee, Knoxville, 105 pp.

Buck, J. D. (1958). Cyclic CO_2 release in insects IV. A theory of mechanism. Biol. Bull. (Woods Hole Marine Biol. Lab.), 114:118-140.

Buck, J. D. (1953). Physical properties and chemical composition of insect blood, In Insect Physiology (K. D. Roeder, ed.), pp. 147-190, Wiley, New York.

Buck, J. D., and Friedman, S. (1958). Cyclic CO_2 release in diapsusing pupae III. CO_2 capacity of the blood: carbonic anhydrase. J. Insect Physiol. 2:52-60.

Bundy, H. F. (1977). Review: carbonic anhydrase. Comp. Biochem. Physiol. 57B:1-7.

Caflish, C. R., and Carter, N. W. (1974). A micro pCO_2 electrode, Anal. Biochem. 60:252-257.

Chauvin, R. (1956). Physiolgic de l'insecta. (W. Junk, The Hague, Netherlands).

Christensen, H. N., and Palmer, G. A. (1974). Enzyme Kinetics, W. B. Saunders Co., Philadelphia.

Coleman, J. E. (1967). Mechanism of action of carbonic anhydrase: Substrate, sulfoxamide and anion binding. J. Biol. Chem. 242: 5212-5219.

Conway, E. J. (1962). Microdiffusion analysis and volumetric error, 5th ed., pp. 201-214, Crosby Lockwood, London.

Conway, E. J., and Byrne, A. (1933). An absorptive apparatus for the microdetermination of certain volatile substances, Biochem. J. 33:419-429.

Demjanowski, S., Galzowa, R., and Roshdestwenska, W. (1932). Wahre aziditat der hamolymphe von Bonbyx mori (L.). Biochem. Z. 247: 386-405.

Duchateau, G., Florkin, M., and Leclercq, J. (1953). Concentration des bases fixes et types de composition de la base total d'hemolymph des insects. J. Arch. Intern. Physiol. 61:518-549.

Ely, M. J., and Jungreis, A. M. (1977). The effects of X-irradiation on egg hatchability and larval survival in the tobacco hornworm, Manduca sexta (Lepidoptera: Sphyngidae). J. Insect Phsiol. 23: 95-101.

Florkin, M. (1935). Sur l'activité anhydrasique du milieu interieur des invertebrates. Arch. Inter. Physiol. 40:283-290.

Florkin, M., and Jeniaux, C. (1974). Hemolymph: Composition, in The Physiology of Insecta. 2nd ed., vol. II (M. Rockstein, ed.) 255-368. Academic Press, New York.

Goh, S., and Phillips, J. E. (1978). Dependence of prolonged water absorption by in vitro locust rectum on ion transport. J. Exp. Biol. 72:25-41.

Hanes, C. S. (1932). Studies on plant amylases 1. The effect of starch concentration upon the velocity of hydroysis by the anylase of germinated barley. Biochem. J. 26:1406-1421.

Harvey, W. R., Haskell, J. A., and Nedergaard, S. (1968). Active transport by the Cecropia midgut III. Midgut potential generated directly by active K-transport. J. Exp. Biol. 48:1-12.

Harvey, W. R. Haskell, J. A., and Zerahn, K. (1967). Active transport of potassium and oxygen consumption in the isolated midgut of Hyalophora cecropia. J. Exp. Biol. 46:235-248.

Harvey, W. R., Wood, J. L., Quatrale, R. P., and Jungreis, A. M. (1975). Cation distribution across the larval and pupal midgut of the lepidopteran Hyalophora cecropia in vivo. J. Exp. Biol. 63:321-330.

Harvey, W. R., and Zerahn, K. (1969). Kinetics and route of active K-transport in the isolated midgut of Hyalophora cecropia. J. Exp. Biol. 50:297-306.

Haskell, J. A., Clemons, R. D., and Harvey, W. R. (1965). Active transport by the Cecropia midgut I. Inhibitors, stimulants and potassium-transport. J. Cellular Comp. Physiol. 65:45-56.

Haswell, M. S. (1967). Carbonic anhydrase in flounder erythrocytes. Comp. Biochem. Physiol. 56A:281-282.

Heimple, A. M. (1956). Further observations on the pH in the gut and the blood of Canadian forest insects. Can. J. Zool. 34:210-212.

Heimple, A. M. (1955). The pH of the gut and blood of the larch sawfly, Pristiphora erichsonii (Hartwig), other insects with reference to the pathogenicity of Baeillus cereus Fr. and Fr.[1]. Can. J. Zool. 33:99-106.

Hevert, F. (1973). Microdetermination of carbonate using a strain-gauge pressure transducer. Pflugers Arch. 344:271-274.

Hikida, R. A., and Jungreis, A. M. (1978). Avesicular exocytosis in integumentary epithelial cells of Hyalophora cecropia during resorption of moulting fluid. The Physiologist 21:54.

Houston, A. H., and McCarty, L. S. (1978). Carbonic anhydrase (Acetazolamide sensitive esterase) activity in the blood, gill, and kidney of the thermally acclimated rainbow trout, Salmo gairdneri. J. Exp. Biol. 73:15-27.

Johnston, J. W., and Jungreis, A. M. (1979). Comparative properties of mammalian and insect carbonic anhydrases: Effects of potassium and chloride on the rate of carbon dioxide hydration. Comp. Biochem. Physiol. 62B:465-469.

Johnston, J. W., and Jungreis, A. M. (1978). A simplified method for assaying micromolar quantities of CO_2. Comp. Biochem. Physiol. 61:469-473.

Johnston, J. W., and Jungreis, A. M. (1978). Comparative aspects of mammalian and insect carbonic anhydrase. The Physiologist 21:467.

Johnston, J. W., and Jungreis, A. M. (1977). Microdiffusional determination of bicarbonate in hemolymph and moulting of Hyalophora cecropia and Manduca sexta. Am. Zoologist 17:861.

Jungreis, A. M. (1979). The physiology of moulting in insects, in Adv. Insect Physiol. 14:109-183.

Jungreis, A. M. (1978). The composition of larval-pupal moulting fluid in the tobacco hornworm, Manduca sexta. J. Insect Physiol. 24:65-73.

Jungreis, A. M. (1974). Physiology and composition of moulting fluid and midgut lumenal contents in the silkmoth, Hyalophora cecropia. J. Comp. Physiol. 88:113-127.

Jungreis, A. M., and Harvey, W. R. (1975). Role of active potassium transport by integumentary epithelium in secretion of larval-pupal moulting fluid during silkmoth development. J. Exp. Biol. 62: 357-366.

Jungreis, A. M., Jatlow, P., and Wyatt, G. R. (1973). Inorganic ion composition of hemolymph of the Cecropia silkmoth: Changes with diet and ontogeny. J. Insect Physiol. 19:225-233.

Jungreis, A. M., and Tojo, S. (1973). Potassium and uric acid content in tissues of the silkmoth, Hyalophora cecropia. Am. J. Physiol. 224:21-26.

Kafatos, F. C. (1968). The labial gland-a salt secreting organ of saturniid moths. J. Exp. Biol. 48:435-453.

Karlmark, B., and Sohtell, M. (1973). The determination of bicarbonate in nanoliter samples. Anal. Biochem. 53:1-11.

Katzenellenbogen, B. S., and Kafatos, F. C. (1971). Proteinases of silkmoth moulting fluid: Physical and catalytic properties. J. Insect Physiol. 17:775-800.

Katzenellenbogen, B. S., and Kafatos, F. C. (1970). Some properties of silkmoth moulting gel and moulting fluid. J. Insect Physiol. 16: 2241-2256.

Kitahara, S., Fox, K. R., and Hagben, C. A. M. (1967). Depression of chloride transport by carbonic anhydrase inhibitors in the absence of carbonic anhydrase. Nature (London) 214:836-838.

Koenig, S. H., and Brown, R. D. (1976). Inhibition of anions of human red cell carbonic anhydrase B: Physiological and biochemical implications. Science 191:745-747.

Levenbook, L. (1950). The physiology of carbon dioxide transport in insect blood. J. Exp. Biol. 27:158-174.

Levenbook, L., and Clark, A. M. (1950). The physiology of carbon dioxide transport in insect blood II. The effect of insect blood on the rate of hydration of CO_2. J. Exp. Biol. 27:175-183.

Levy, R. I., and Schneiderman, H. A. (1958). An experimental solution to the paradox of discontinuous respiration in insects. Nature 182:491-493.

Little, C., and Ruston, G. (1969). A method for determining total carbon dioxide in small volumes of liquid. J. Exp. Biol. 52:395-400.

Locke, M. (1976). The role of plasma membrane plaques and golgi complex vesicles in cuticle deposition during the moult-intermoult cycle. In: The Insect Integument (H. R. Hepburn, ed.), pp. 237-258. Amsterdam: Elsevier Publishing Co.

Locke, M. (1974). The structure and formation of the integument in insects. In: The Physiology of Insecta, Vol. VI, 2nd Edition (M. Rockstein, ed.), pp. 123-213. New York: Academic Press.

Locke, M., and Krishnan, N. (1973). The formation of the ecdysial droplets and the ecdysial membrane in an insect. Tissue and Cell 5: 441-450.

Lockstein, R. A., Epstein, D. S., Joestein, M., Jimenez, M., Ambrosino, B., and Bais, D. (1978). Fluid regulation in metamorphosing insects. The Physiologist 21 (#4):72.

Maddrell, S. H. P. (1971). The mechanisms of insect excretory systems. Adv. Insect Physiol. 8:199-331.

Maddrell, S. H. P. (1969). Secretion by the malphighian tubules of rhodnius: The movements of ions and water. J. Exp. Biol. 51:71-97.

Maffly, R. H. (1968). A concuctrimetric method for measuring micromolar quantities of carbon dioxide. Anal. Biochem. 23:252-262.

Maren, T. H., Rayburn, C. S., and Liddell, N. E. (1976). Inhibition of anions of human red cell carbonic anhydrase B: Physiological and biochemical implications. Science 191:469-472.

Maren, T. H. (1967). Carbonic anhydrase: Chemistry, physiology and inhibition. Physiol. Review 47:595-781.

Merryfield, M. (1979). Effects of bicarbonate on rat liver P-enolpyruvate carboxy kinase (PEPCK) ferroactivator. Federation Proceedings, vol. 38 (#3), Part I, p. 674.

Obrink, K. J. (1955). A modified conway unit for microdiffusional analysis. Biochem. J. 59:34-46.

Passonneau, J. V., and Williams, C. M. (1953). The moulting fluid of the Cecropia silkmoth. J. Exp. Biol. 30:545-560.

Pita, J. C. (1969). Potentiometric determination of carbon dioxide partial pressure and pH in ultramicro volumes of biological fluids. Anal. Chem. 41:273-278.

Phillips, J. (1977). Problems of H_2O transport in Insects, in Water Relations in Membrane Transport in Plants and Animals (ed. by A. M. Jungreis, T. K. Hodges, A. Kleinzeller, and S. G. Schultz), pp. 333-353, New York: Academic Press.

Phillips, J., Williams, D., Prince, D., and Spring, J. (1977). Ion transport across insect rectal epithelium under short-circuit conditions. Proceedings of the International Union of Physiological Sciences, vol. Xii, p. 595.

Pocker, Y., and Stone, J. T. (1967). The catalytic versatility of erythrocyte carbonic anhydrase. III. Kinetic studies of the enzyme-catalyzed hydrolysis of p-nitrophenyl acetate. Biochemistry 6: 668-678.

Pocker, Y., and Tanaka, N. (1978). Inhibition of carbonic anhydrase by anions in the carbon dioxide-bicarbonate system. Science 199: 907-909.

Prop, F. J. A. (1954). A micro-respirometer for simultaneous measurement of O_2 consumption and CO_2 production and its use in determining the respiratory quotient of tissue cultures. Exp. Cell. Research 7:303-317.

Riddiford, L. M. (1968). An artificial diet for Cecropia and other saturniid silkworms. Science 160:1461-1462.

Riepe, M. E., and Wang, J. H. (1967). Elucidation of the catalytic mechanism of carbonic anhydrase. J. Am. Chem. Soc. 89:4229.

Rudolph, S. A. (1979). Effects of frog blood plasma components and bicarbonate on cation fluxes in frog erythrocytes. Federation Proceedings, vol. 38 (#3), Part 3, p. 243.

Schultz, T. W., and Jungreis, A. M. (1977). Origin of the short circuit decay profile and maintenance of the cation transport capacity of the larval lepidopteran midgut in vitro and in vivo. Tissue and Cell 9 (2):255-272.

Severinghaus, J. W., and Bradley, A. F. (1958). Electrodes for blood pO_2 and pCO_2 determination. J. Applied Physiol. 13:515-520.

Snell, F. M. (1960). Electrometric measurement of CO_2 and bicarbonate ion. J. Applied Physiol. 15:729-732.

Spring, J., Hanrahan, J., and Phillips, J. (1978). Hormonal control of chloride transport across locust rectum. Can. J. Zool. 56:1879-1882.

Turbeck, B. O., and Foder, F. (1970). Studies on a carbonic anhydrase from the midgut epithelium of larvae of lepidoptera. Biochem. Biophys. Acta 212:139-149.

Turbeck, B. O., Nedergaard, S., and Kruse, H. (1968). An anion-stimulated adenosine triphosphatase from the potassium-transporting midgut of the larva of Hyalophora cecropia. Biochim. Biophys. Acta 163:354-361.

Ussing, H. H., and Zerahn, K. (1951). Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. Acta. Physiol. Scand. 23:110-127.

Van Broggen, J. T., and Scott, J. C. (1962). Microdetermination of carbon dioxide. Anal. Biochem. 3:464-471.

Van Slyke, D. D., and Neill, J. M. (1924). The determination of gases in blood and other solutions of vacuum extraction and manometric measurement. J. Biol. Chem. 523-573.

Vurek, G. G., Warnock, D. G., and Corsey, R. (1975). Measurement of picomole amounts of carbon dioxide by calorimetry. Anal. Chem. 47: 765-767.

Wilbur, K. M., and Anderson, W. G. (1948). Electrometric and colormetric determination of carbonic anhydrase. J. Biol. Chem. 176:147-154.

Williams, F. W., Woods, F. J., and Umstead, M. E. (1972). Determination of carbon dioxide in the parts-per-million range with gas chromatography. J. Chromatogr. Sci. 10:570-572.

Williams, D., Phillips, J. E., Prince, W. T., and Meredith, J. (1978). The source of short-circuit current across locust rectum. J. Exp. Biol. 77:107-122.

Wistrand, P. J., and Rao, S. N. (1968). Immunologic and kinetic properties of carbonic anhydrases from various tissues. Biochim. Biophys. Acta 154:130-144.

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

Wyatt, G. R. (1961). The biochemistry of insect hemolymph. Ann. Review Entomol. 6:75-102.

VITA

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