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Some Aspects of Amino Acid Regulation in the Tobacco Hornworm, *Manduca sexta*

J. Michael Henry
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To the Graduate Council:

I am submitting herewith a thesis written by J. Michael Henry entitled "Some Aspects of Amino Acid Regulation in the Tobacco Hornworm, *Manduca sexta*." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

Arthur M. Jungreis, Major Professor

We have read this thesis and recommend its acceptance:

James M. Liles

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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and recommend its acceptance:

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Harold Ufer

Accepted for the Council:

L. Evans Roth
Vice Chancellor
Graduate Studies and Research

SOME ASPECTS OF AMINO ACID REGULATION IN THE TOBACCO HORNWORM, *MANDUCA SEXTA*

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

J. Michael Henry

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ABSTRACT

The nature and roles of the high free amino acid levels found in the haemolymph of insects is poorly understood. In order to elicit aspects of the regulation and metabolism of haemolymph free amino acids, we have measured the concentrations, half-lives, turnover rates and rates of incorporation into haemolymph proteins for eight amino acids in the tobacco hornworm, Manduca sexta immediately prior to and throughout the larval-pupal transformation. Throughout this period the concentrations of alanine, arginine, glutamate, histidine, leucine, lysine, proline and valine each exhibited stage specific variations seemingly independent of blood volumes and levels of other amino acids. Half-lives for these amino acids also exhibited reproducible stage specific variations indicating independent regulation. Half-lives demonstrated a wide range of utilization (2-170 hours) and do not change in parallel fashion for the amino acids investigated. Conclusions drawn from this study are that a) amino acids are regulated and b) although some amino acids such as histidine appear to be stored in haemolymph, most are dynamically maintained and must be major determinants of insect homeostasis.

TABLE OF CONTENTS

LIST OF FIGURES

I. INTRODUCTION

The concentrations of amino acids in tissues and haemolymph from a wide range of insect orders exemplified by numerous species at selected stages in development have been published (see citations in Florkin, 1959; Wyatt, 1961; Florkin and Jeuniaux, 1974). In general, these studies have been toxonomic rather than physiological in outlook in that they describe the distribution of amino acids at a single point in time (see Sutcliffe, 1963). Amino acid metabolism and regulation in insects are poorly described (see however Auclair, 1959; Whitehead, 1969; Desai and Kilby, 1958; Price, 1961; Chen and Bachmann-Diem, 1965), with few measurements having been made of amino acid fluxes through haemolymph or whole body pools (see Levenbook and Dinamarca, 1966; Collett, 1976; Irving, Osborne and Wilson, 1979). The concentration changes of amino acids in haemolymph during development are recorded without references to quantity (i.e., as the contribution of the blood volume to the unit body weight changed?) or metabolic flux (turnover).

While the static nature of haemolymph sugar is often implicit in the many reports appearing on the subject in the literature, that for free amino acids is quite explicit. In a volume on insect physiology, Patton (1963) writes:

> ...the values (of amino acids) for the blood of immature stages have been selected, the reason being that these values are more likely to remain constant than those of adult blood....
>
> One characteristic of amino acidemia in the blood of insects is its constancy...free amino acids found in the blood in the largest quantities are not those amino acids essential to the nutrition of the insect. From this it can be suggested that the high amino acid concentration in the blood of insects represents the storage of nitrogenous materials that can be drawn upon, according to the needs of the tissues...orit may indicate that an excess of amino acids are produced from the diet and that the amino acids are stored in blood until they can be eliminated...

This view was further supported even by Wigglesworth (1965) who, in discussing haemolymph amino acids, included examples that demonstrate the passive nature and putative storage functions of haemolymph:

> There seems to be no exact regulation of the amino acid patterns: the hemolymph of the aphid Megoura contains the same amino acids as honey dew and in similar concentrations (Ehrhardt, 1962). If Rhodnius is fed on horse serum with added alanine, alanine appears in the haemolymph within a few minutes and in the urine in half an hour, but the high level in the haemolymph persists for weeks (Harrington, 1961).

In this study, the rates of turnover and the half-lives of eight haemolymph amino acids were determined in haemolymph from the tobacco hornworm, Manduca sexta, immediately before and throughout the larval-pupal transformation. The questions I posed were:

1. whether these amino acids are regulated statically or dynamically (i.e., do haemolymph pools represent sinks in which levels change only with tissue needs, or are they in constant dynamic interaction with tissues),

2. whether stage specific changes in amino acids occur independently, and

3. to what extent are the changing titers of free amino acids reflections of changes in absolute and relative haemolymph volumes.

II. MATERIALS AND METHODS

General Procedures

Tobacco hornworms (Manduca sexta L.) used in these experiments were laboratory reared on the wheat germ-casein diet of Bell and Joachim (1976) as modified by Baumhover, et al. (1977) under a 16L:8D photoperiod (photophase initiated at 0600 AZT) and 60% relative humidity. Hornworms were staged according to the criteria summarized in Jungreis (1979). Mature feeding fifth instar larvae were first studied about 24 hours after excretion of frosted frass (average weight = 9.2 ± .3 gm), and throughout pharate pupal development (Jenkin and Hinton, 1966) from the onset of wandering or the pink stripe stage (Nijout and Williams, 1974) to the day before the larval-pupal ecdysis (pink stripe + 4 days). Hornworms were administered amino acids at 1400 AZT. Following various periods of incubation, haemolymph and moulting fluid from insects that had been chilled in crushed ice for 5-10 minutes were collected following procedures outlined in Jungreis (1978). (Moulting fluid was collected on Pink Stripe + 3-4 days only.) Fluids collected from individual insects were always treated separately.

Amino Acid Analysis

The concentrations of amino acids were determined in fifth instar larvae and on each day of the larval-pupal transformation (LPT). Fluids were centrifuged at up to 1000 x g for 5-15 seconds to remove cells, cellular debris, and insoluble phenylthiourea. Aliquots (90 microliters) were transferred to clean microcentrifuge tubes and 10 microliters of 30% (w:v) 5-sulfosalicylic acid, SSA, (Pierce Chemical Corp. Rockford, Il), pH 2.1, were added to precipitate the protein. Ten minutes after addition of SSA (final concentration of 3%), samples were recentrifuged at 8000 x g for 2 minutes at room

temperature and separate 50 microliter aliquots of the supernatant fractions were pooled and frozen at -25°C until analyzed. Haemolymph from three to six individuals were pooled at each stage of development. During secretion and resorption of moulting fluid, haemolymph and moulting fluid, respectively, were collected from 75 individual animals (equal numbers of early, middle and late moulting animals; pink stripe +2, 3 and 4 days; see Jungreis, 1978); pooled (taking an equal contribution from each) and assayed. Prior to chromatographic analysis, residual precipitate was removed from pooled supernatants by centrifugation at 12,000 x g for 5 minutes at 23°C. Sample pH's were then remeasured and if necessary adjusted to pH 2.1 by addition of 4% (w:v) lithium hydroxide or 3% SSA. Aliquots of 5-40 microliters of the pooled supernatants were then analyzed in a Durrham D-500 Amino Acid Analyzer by the five-buffer procedure of Lee (1974) as described in Jungreis and Omalianowski (1980). The spectra of the eluted samples were compared with 5 microliters of a standard solution containing at concentrations of 1 mM approximately 75 amino acids, amino sugars, amino acid analogues and urea cycle intermediates. Agreements between the two sample volumes and between pooled samples at the same stage in development were within 4% for the amino acids investigated. Haemolymph amino acid concentrations previously determined only at selected stages in M. sexta development (Taylor, 1979; Riddiford, 1980) were confirmed and extended by this analysis.

Determination of Half-lives

Half-lives and turnover of selected amino acids in the haemolymph were determined using ^{3}H (specific activity 5-16 Ci/mMole) or ^{14}C (specific activity 350-400 mCi/mMole) labelled amino acids (New England Nuclear). Alanine, arginine, glutamine (glutamate), histidine, leucine, lysine, proline and

valine were studied because they are present in significant (mMolar) concentrations in the haemolymph. On each day of development, fifteen animals were injected with 0.2 microcuries ^{3}H-, or 0.1 microcuries ^{14}C-labelled tracer (25-65 µl). Haemolymph was then separately collected from five animals after three, six and nine hours. The time of initial collection, 3 hours, was chosen both to allow for complete equilibration of the label in haemolymph (see Williams-Boyce and Jungreis, 1980), and because other amino acids were found to have half lives on the order of 3-5 hours (Jungreis, unpublished). The three hours duration periods between sample collections were chosen to more accurately measure turnover. Longer collection times could not be employed due to continued development in the hornworms administered amino acids.

Blood was collected from insects by making an incision above the dorsal aorta through the dorsum. Drops of haemolymph were collected on parafilm over ice and phenylthiourea (PTU) was added to prevent melanization. Haemolymph from each animal was then centrifuged to remove haemocytes or undissolved PTU. Haemocyte numbers in M. sexta increase during the larval-pupal transformation (Jungreis and Omalianowski, 1980). However, the contribution of haemocyte free amino acids to the total amino acid pool can be neglected because maximal cell volumes are only 0.006-0.03% of the total haemolymph volume (Jungreis and Omalianowski, 1980). Haemoplasm supernatants were decanted and separated into two aliquots. One aliquot was used to determine total isotope per unit volume, while the second aliquot was deproteinated with SSA. Protein free supernatants were frozen at -25°C prior to analysis for radioactivity.

The proportions of unmetabolized amino acids in relation to total radioactivity in the deproteinated fractions were determined after Haer (1969) by thin layer chromatography on Gelman ITLC, type SA, thin layer plates with

either n-butanol:glacial acetic acid:water (4:1:1, v:v:v) or propanol:water (7:3, v:v) solvent systems. Deproteinated fractions of haemoplasm were concentrated at 50-75°C and 20psi vacuum until 100 µl possessed ca. 6000 dpm. Fifty microliters of each amino acid sample were then spotted five microliters at a time to individual thin layer plates. Appropriate radioactive amino acid standards (New England Nuclear) were then spotted on each plate. Standards were also added to deproteinated haemoplasm and concentrated at 75°C, 20psi to a specific activity equal to that of collected samples. The thin layer plates were allowed to run to a 15 cm solvent front and R_f's determined from amino acids standards were employed to localize the unmetabolized amino acids. Percentages of unmetabolized amino acid were calculated from the following formula:

$$\% \text{ amino acid remaining} = \frac{\text{dpm recovered as amino acid}}{\text{total dpm in 50 µl}} \times \frac{\text{total dpm in 50 µl standard}}{\text{dpm recovered in standard}} \times 100\% \qquad \text{Eq. 1.}$$

The percentages obtained were then used to calculate amino acid specific activities in samples counted for half-life determinations. Incorporation of amino acids into haemoplasm soluble protein was measured with the aid of Equation 2:

$$\% \text{ amino acid incorporated into protein} = \frac{\text{dpm in 50 µl of whole hemoplasm} - \text{dpm in 50 µl of deproteinated hermoplasm}}{\text{dpm in 50 µl of whole haemoplasm}} \times 100\% \qquad \text{Eq. 2.}$$

The pattern of amino acid uptake from haemolymph was found to fit an exponential equation:

$$N = N_o e^{-kt} \qquad \text{Eq. 3.}$$

where N_o is the initial dpm concentration, t is the time since injection of

label, and k is the decay constant (see Figure 1). By setting $N = \frac{1}{2} N_o$, a half-life can be calculated. Data was log transformed to facilitate linear regression analysis.

$$\ln N/N_o = -kt \qquad \text{Eq. 4.}$$

Linear regression analysis using Student's 't' distribution and the method described by Sokal and Rohlf (1969) was performed on this transformed data for each amino acid at every stage of the larval-pupal transformation (Figure 2). Under ideal circumstances, an amino acid should go through at least one half-life during the period of measurement. However, it was not feasible to follow this procedure on amino acids with long half-lives, such as histidine, as estimated half-lives greatly exceeded the developmental period (24 hours) under measurement. Failure to include at least one biological half-life during the 9 hour period of measurement endows the calculated half-life with a substantial 95% confidence interval about the regression line of up to 25%. The information obtained for such amino acids is useful in that a rough estimate for the half-life is still obtained. Quantitative measurements of turnovers for each amino acid were calculated using half-lives from Equation 4, their haemolymph concentrations and a knowledge of the blood volume (Williams-Boyce and Jungreis, 1981).

$$\text{Turnover rate} = \frac{\text{concentration of amino acid} \times \text{stage specific blood volume}}{\text{half-life} \times 2} \qquad \text{Eq. 5.}$$

Figure 1. Typical plot of data to the exponential decay equation (Equation 3). Shown here is the fit of ^{14}C-proline data collected from pink stripe + one day and fitted to an equation $N = 2613\ e^{-0.11t}$. At three, six and nine hours, means ± 95% confidence intervals for five different animals are plotted (total n = 15).

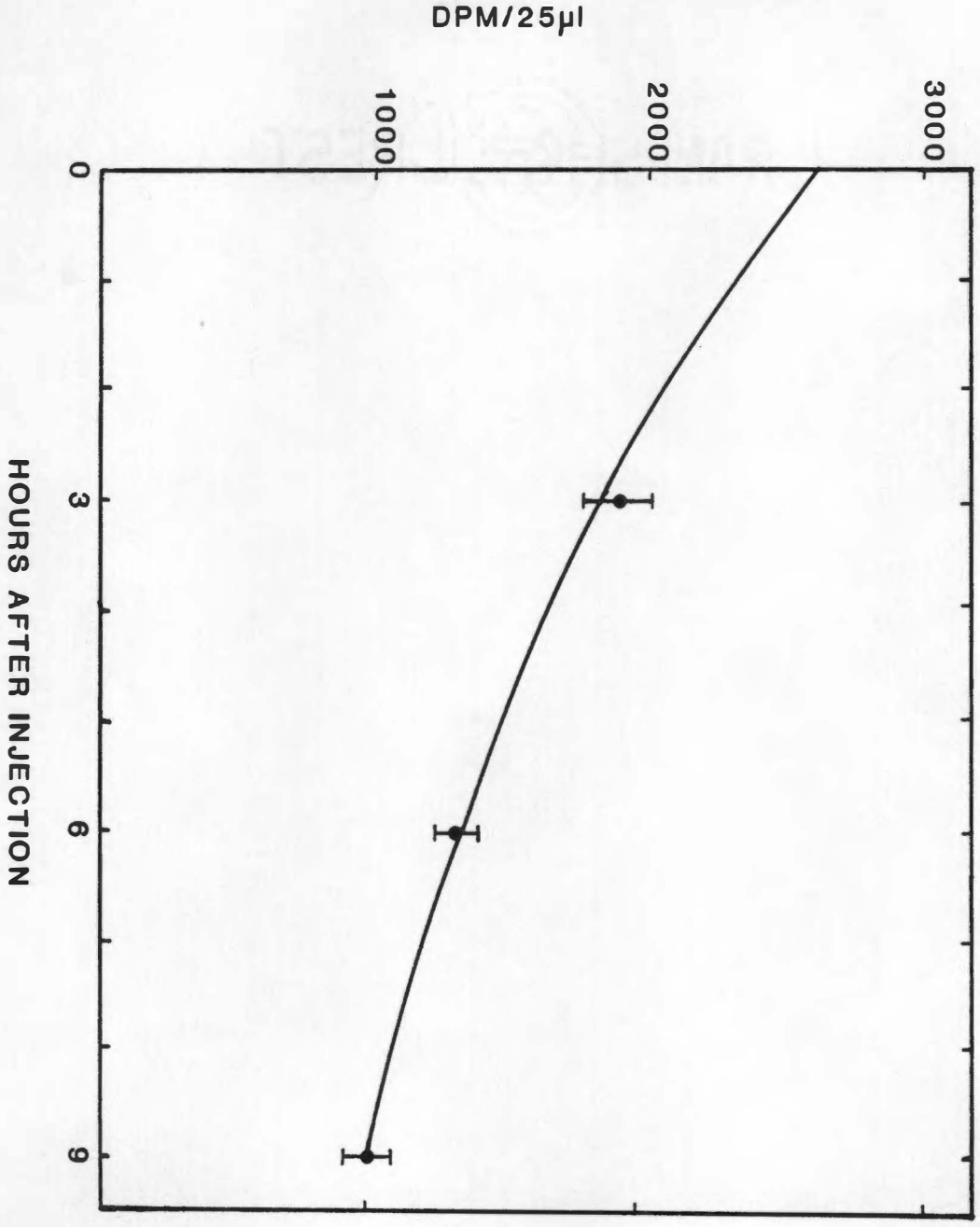

Figure 1

Figure 2. Fit of log transformed data for ^{14}C-proline on pink stripe + 1 day to Equation 4. 95% confidence intervals are shown for the three, six and nine hour time points (total n = 15).

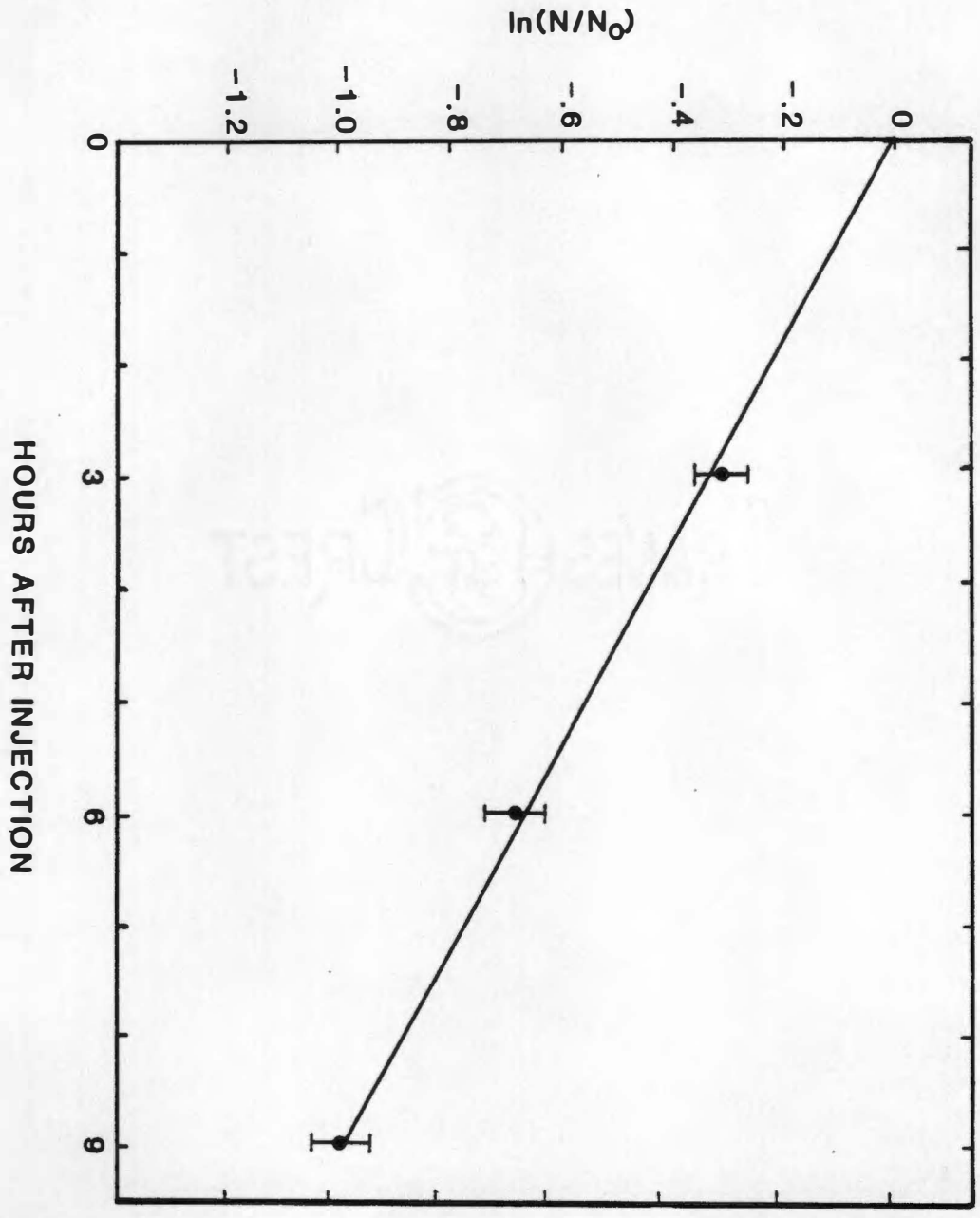

Figure 2

III. RESULTS

Amino Acid Analysis

The concentrations of amino acids in haemolymph were measured on each day of the larval-pupal transformation (Figures 3 and 4). The concentrations of alanine, leucine, glutamate, histidine, proline and lysine all exhibited substantive declines between the feeding fifth larval and the pink stripe stages. Valine and arginine did not decline. Such declines were not expected, as the haemolymph volume does not change significantly at this time (Williams-Boyce and Jungreis, 1980). Towards the end of the LPT, concentrations of the respective amino acids increase until levels equal to or exceeding those characteristic of feeding larvae are again present. Since normalized blood volumes decline some 50% during the LPT (3.97 to 2.00 mls, Williams-Boyce and Jungreis, 1980), total quantities of free amino acids in haemolymph were calculated at each intermediate stage of the LPT to provide a more accurate picture of metabolic change (Table I). Expressed as quantities of amino acid per animal, the two most notable features of these pool size measurements are that each amino acid exhibited appreciable day-to-day variations, and further that these variations appeared independent of one another. Histidine, the amino acid present at a concentration of 18 mM, was invariant during the LPT, with daily quantities of 50 micromoles/animal consistently recorded. Following an initial decline between the feeding larval and PS stages total quantities per animal of lysine and alnine remained constant until the pink stripe + 3 days and pink stripe + 2 days, respectively. On the other hand, proline exhibited appreciable variance throughout the LPT. After an initial decline from 2.2 to 0.4 micromoles/animal at the PS stage, glutamate quantities increased progressively until a 30 fold increase was noted

Figure 3. Concentrations of valine, alanine, leucine and glutamate in haemolymph of *Manduca sexta* in feeding larvae and in pharate pupae throughout the larval-pupal transformation. Values are means + standard deviations for 5 pools of hemolymph collected from 3-6 animals per pool in feeding larvae, pink stripe and pink stripe + one day stages; 2 pools from 5-6 animals on pink stripe + 2 or 3 days; and 2 pools from 15 or 75 animals per pool on pink stripe + 4 days. A = valine B = alanine C = leucine D = glutamate

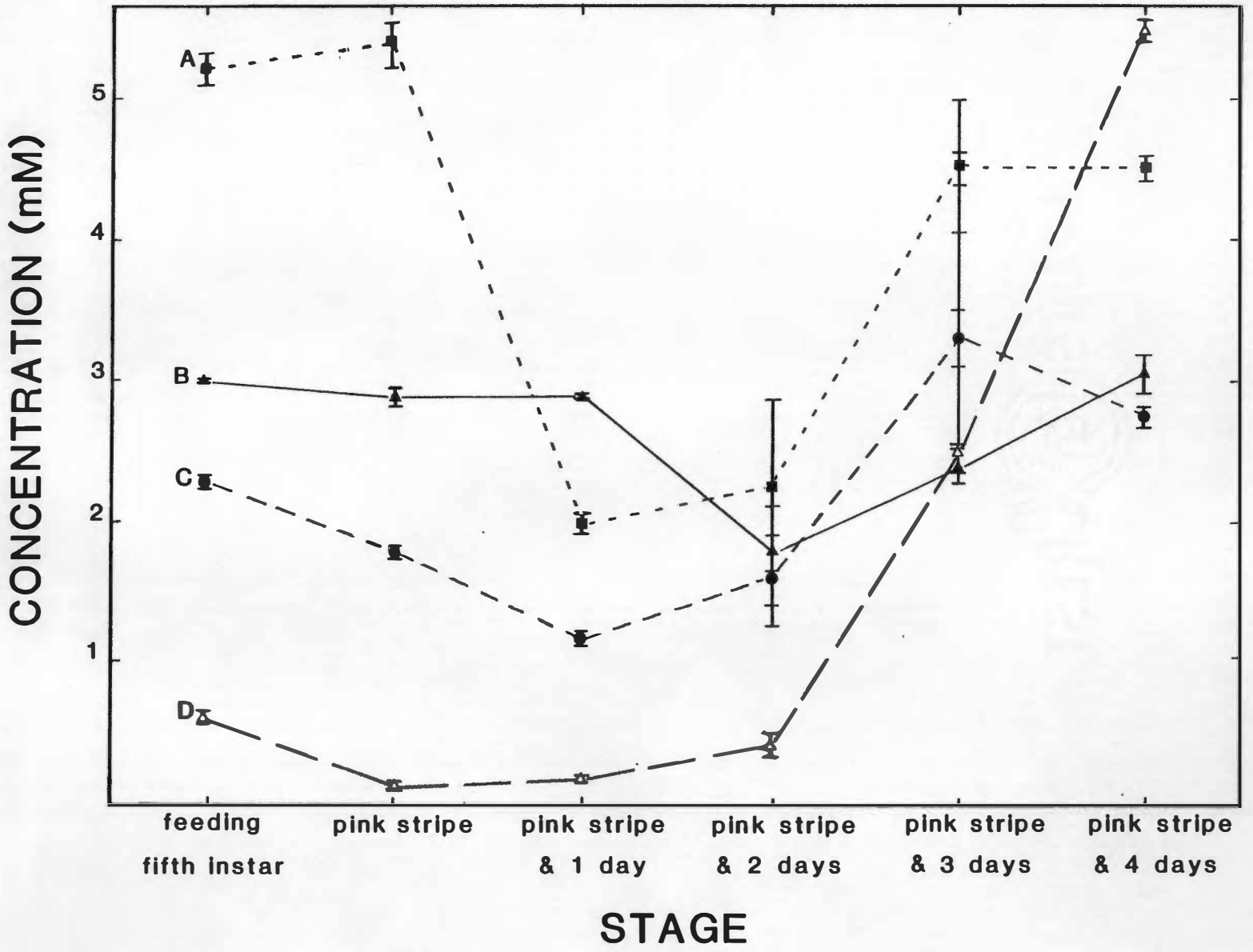
CONCENTRATION (mM)
5
4
3
2
1
A
B
C
D
feeding
fifth instar
pink stripe
pink stripe
& 1 day
pink stripe
& 2 days
pink stripe
& 3 days
pink stripe
& 4 days
STAGE

Figure 3

Figure 4. Concentrations of histidine, proline, lysine and arginine in haemolymph of M. sexta collected from feeding larvae and in pharate pupae throughout the larval-pupal transformation. See legend Figure 3. A = histidne B = proline C = lysine D = arginine

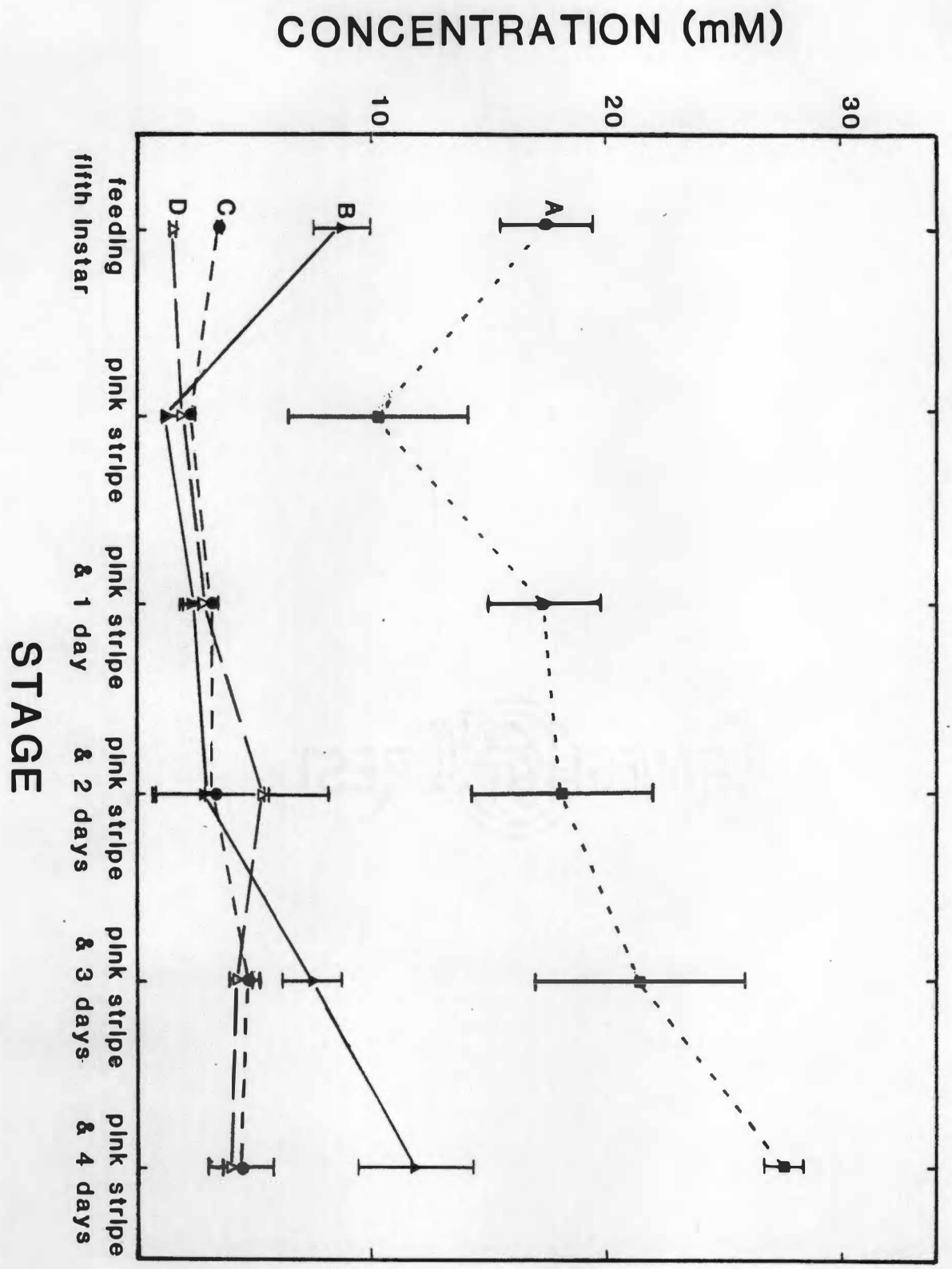
CONCENTRATION (mM)
10
20
30
A
B
C
D
feeding
fifth instar
pink stripe
pink stripe & 1 day
pink stripe & 2 days
pink stripe & 3 days
pink stripe & 4 days
STAGE

Figure 4

immediately before the larval-pupal ecdysis (Table, I Jungreis and Omalianowski, 1980). The quantity of proline in haemolymph dropped from its maximum of 31 micromoles per animal in feeding larvae to only 4.2 micromoles at the pink stripe stage. Since proline utilization has been implicated as a primary substrate for intense energy requiring activities such as flight (Hargrove, 1975; Brouwers and DeKort, 1978; Weeda, 1979; Mordue and DeKort, 1977), its dissappearance from haemolymph at this time could be related to enhanced energy expenditures associated with wandering. In contrast to the other amino acids studied in M. sexta, the quantity per animal of arginine increased between the feeding larval and the pink stripe + 2 days stages (5.3 to 14.2 micromoles/animal), whereupon it began to decline until a value of 7.8 micromoles per animal was measured on pink stripe + 4 days.

Half-life and Turnover Determination

Stage specific half-life determinations are independent of blood volume or pool size and are unaffected by overestimation or underestimation of these factors. The calculated half-lives of eight amino acids measured before and throughout the larval-pupal transformation in M. sexta reflect the changing patterns of metabolic activity (Figures 5-8). During the LPT, half-lives generally remained either constant (valine, leucine) or showed a tendency towards increase (e.g. arginine). Alanine is present in hemolymph at relatively constant quantities, yet it always had a half-life under 2 hours, with only 5% of the initial label remaining after 3 hours. These results confirm studies of rapid alanine turnover in whole bodies of Phormia (Levenbook and Dinamarca, 1966) and in haemolymph of Calliphora (Collett, 1976). Levenbook and Dinamarca (1966), who detected substantial label released as $^{14}CO_2$, proposed a metabolic relationship linking $^{14}CO_2$ production to alanine catabolism and

Table 1. Quantities of amino acids in haemolymph of M. sexta throughout the larval-pupal transformation. Quantities were calculated from known concentrations and stage specific blood volumes (c.f. Williams-Boyce & Jungreis, 1980).

	Feeding Fifth Instar	Pink Stripe	Pink Stripe +1 Day	Pink Stripe +2 Days	Pink Stripe +3 Days	Pink Stripe +4 Days
Alanine	11.0	9.0	9.3	5.0	6.2	6.1
Arginine	5.3	7.6	9.0	14.2	10.8	7.8
Glutamate	2.2	0.4	0.5	1.1	6.3	12.4
Histidine	63.1	40.1	55.4	50.0	55.6	55.0
Leucine	8.3	7.1	3.7	4.4	8.7	5.5
Lysine	13.3	8.3	8.1	8.0	12.0	7.8
Proline	31.3	4.2	7.7	8.1	19.2	23.8
Valine	19.0	21.4	6.4	6.2	11.8	9.0
Stage Specific Volume of Haemo-lymph (mls)	3.65	3.97	3.20	2.76	2.60	2.00

Figure 5. Half lives for proline and leucine in feeding fifth instar larvae and in pharate pupae throughout the larval-pupal transformation. Half lives were determined using individually collected and analysed haemolymph from 15 animals at each stage of the larval-pupal transformation and at the feeding larval stage. Values are Means ± standard deviations. A = proline B = leucine

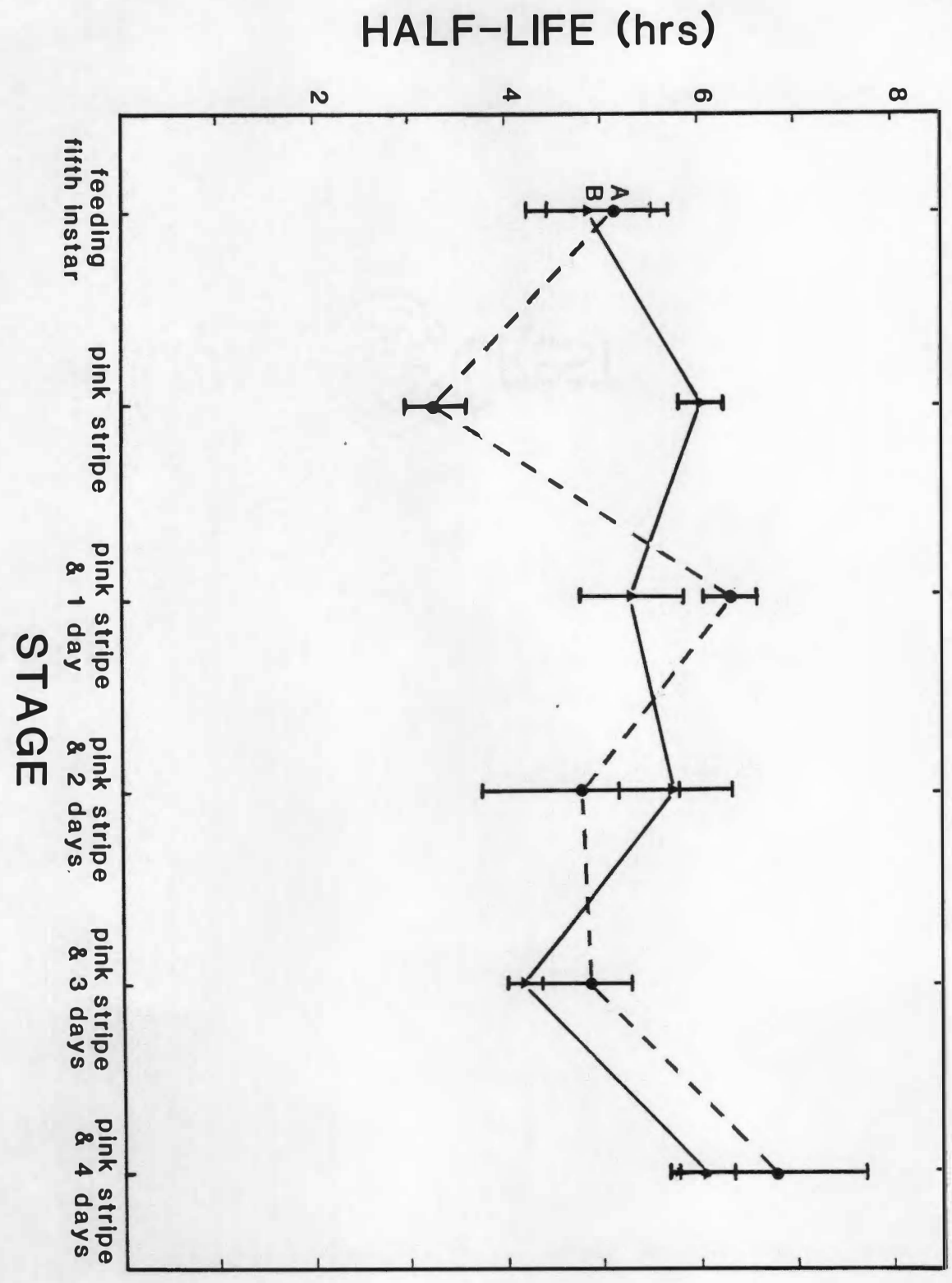
HALF-LIFE (hrs)
2
4
6
8
A
B
feeding fifth instar
pink stripe
pink stripe & 1 day
pink stripe & 2 days
pink stripe & 3 days
pink stripe & 4 days
STAGE

Figure 5

Figure 6. Half lives for histidine in feeding fifth instar larvae and pharate pupae throughout the larval-pupal transformaiton. See legend for Figure 5.

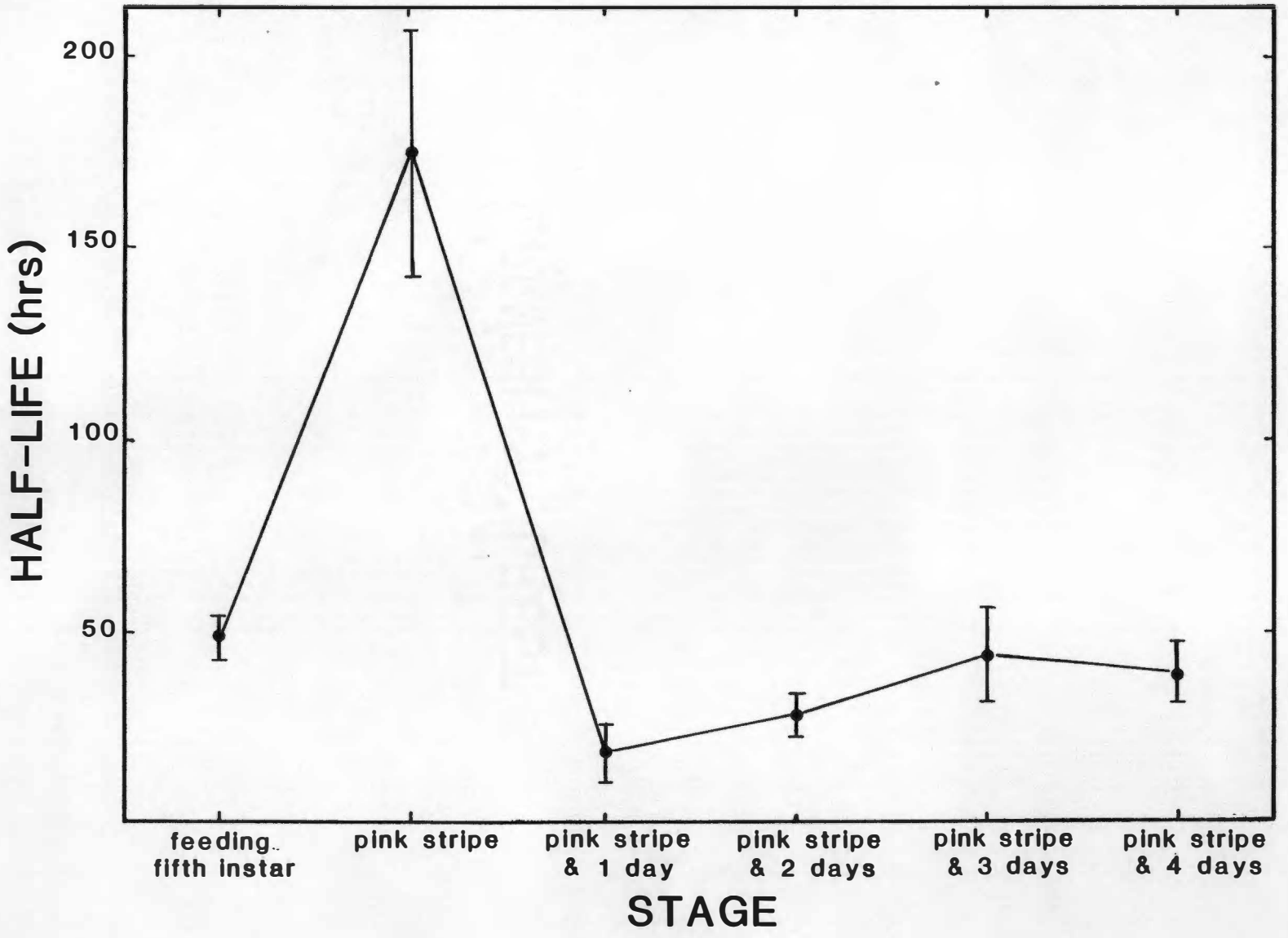
HALF-LIFE (hrs)
200
150
100
50
feeding fifth instar
pink stripe
pink stripe & 1 day
pink stripe & 2 days
pink stripe & 3 days
pink stripe & 4 days
STAGE

Figure 6

Figure 7. Half lives for valine and glutamate in feeding fifth instar larvae and pharate pupae throughout the larval-pupal transformation. See legend for Figure 5. A = valine B = glutamate

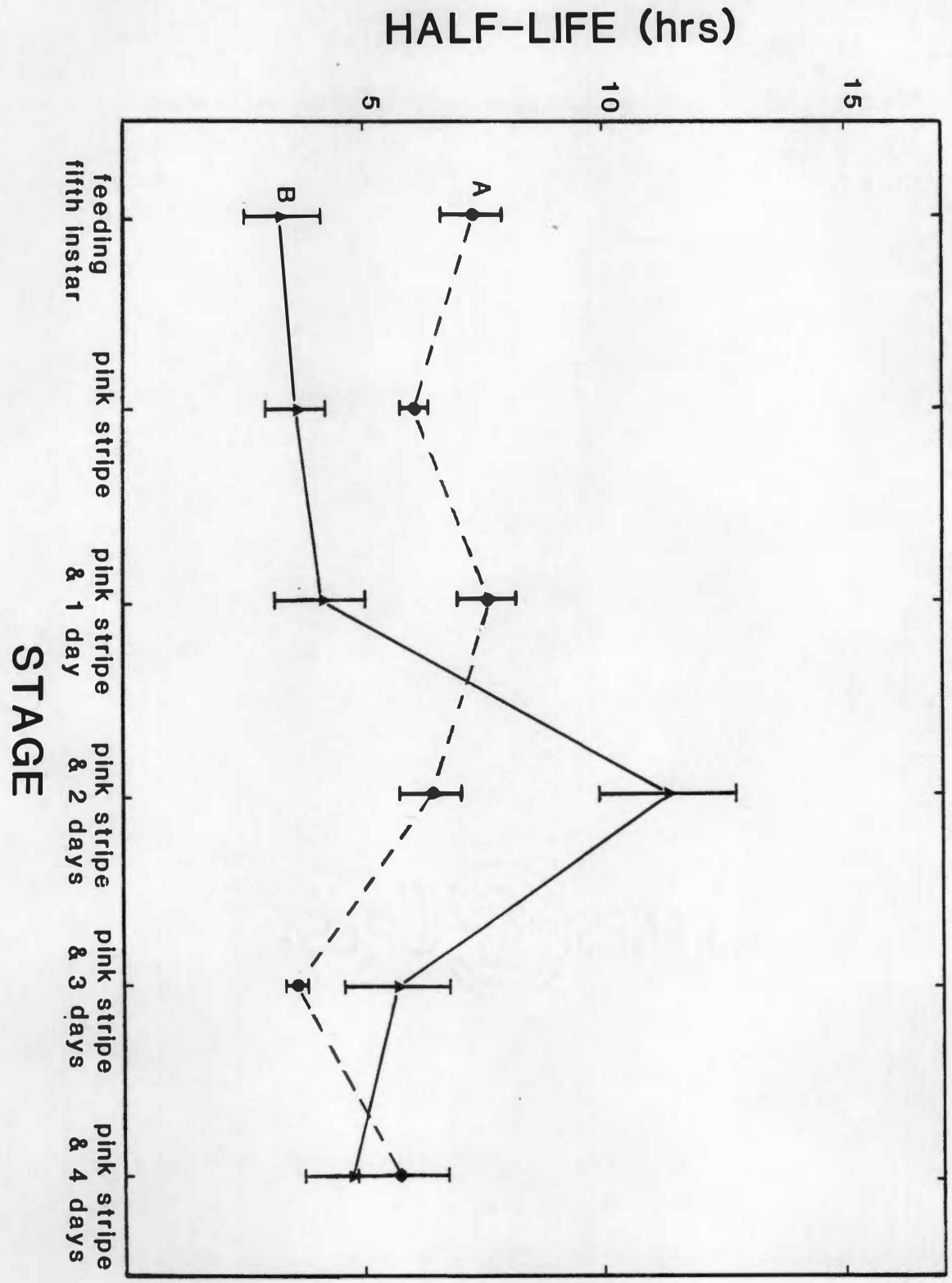
HALF-LIFE (hrs)
5
10
15
A
B
feeding
fifth instar
pink stripe
pink stripe
& 1 day
pink stripe
& 2 days
pink stripe
& 3 days
pink stripe
& 4 days
STAGE

Figure 7

Figure 8. Half lives for arginine and lysine in feeding fifth instar larvae and pharate pupae throughout the larval-pupal transformation. See legend for Figure 5. A = arginine B = lysine

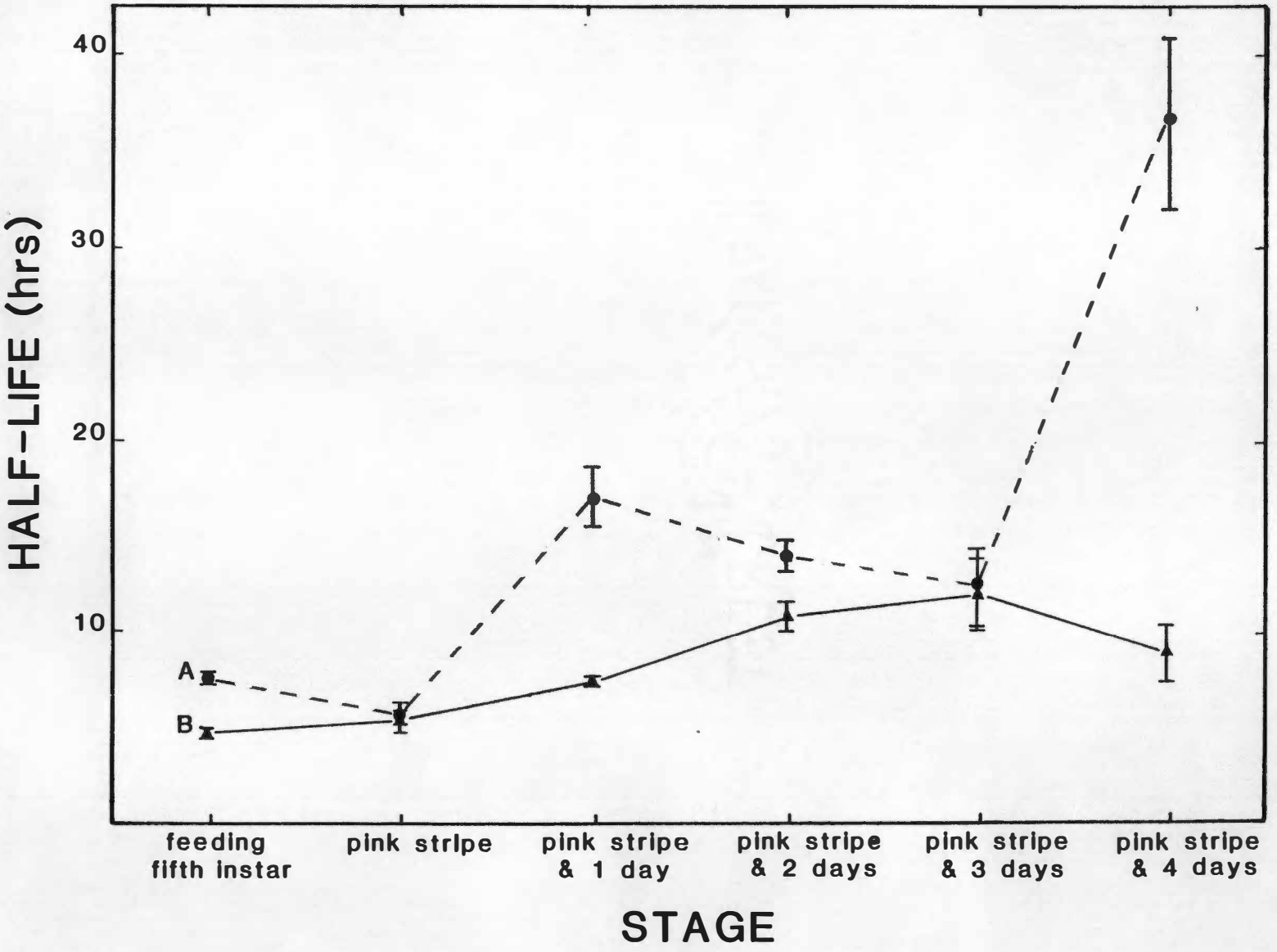
HALF-LIFE (hrs)
40
30
20
10
A
B
feeding fifth instar
pink stripe
pink stripe & 1 day
pink stripe & 2 days
pink stripe & 3 days
pink stripe & 4 days
STAGE

Figure 8

O_2 uptake. Using Calliphora adults, Collett (1976) could recover only 2% of the initial free alanine label in haemolymph after 2 hours. In contrast, histidine, had an absurdly long half-life throughout the LPT (up to 170 hours), indicating storage in hemolymph and a putative role in osmotic regulation. In fact, large quantities of histidine are excreted in meconium of M. sexta following adult eclosion (Levenbook, Hutchins and Bauer, 1971). A recent study on osmoregulation in crickets by Woodring and Blakeney (1980), provided evidence in support of the contention that only a small number of the available amino acids are actually utilized for this purpose. Rather, only those amino acids present in greatest quantities would serve in an osmoregulatory capacity. In Acheta, proline was found to serve this function. Histidine, quantitatively the largest contributor to total free amino acids in haemolymph of M. sexta, could function in an osmoregulation capacity, since its half-life in relationship to other haemolymph amino acids is long. As one would predict from the very low quantities present in haemolymph at this time (0.4-1.1 micromoles per animal), glutamate exhibited a short half-life (3 hours) during the first three days of the LPT. When the concentration of glutamate rose initially at PS+2 days, it was accompanied by a rise in the half-life to 11 hours. However, as its quantity in blood continued to rise on PS+3 or PS+4 days, the half-life of glutamate fell to only 4.5 hours, suggesting a major shift in glutamate utilization. Increases in the quantity per animal of glutamate indicates a temporary decline in glutamate utilization on PS+2 days, with its consequent increase in the haemolymph glutamate pool size. Coupled with a much shorter half-life on PS+3 days, it becomes clear that glutamate utilization must increase at least four fold. Turnover calculations demonstrate a ten fold increase on PS+3 days and a 25 fold increase on PS+4 days. Half-lives of other amino acids do not follow predictable patterns, each occurring independently of each

other. Proline, valine and leucine half-lives remain relatively constant throughout the LPT at around 5-7 hours. Lysine and arginine half-lives increased slightly as ecdysis approached. In Table 2, half-lives for these same amino acid are recalculated as turnover rates using Equation 5.

Turnover calculations, unlike half-life determinations, can be severely affected by errors in estimation of both blood volume and concentration. Thus care was taken both in staging and rearing of animals to minimize these effects. Interconversion of amino acids and generation of metabolic products in haemolymph may produce a significant underestimation of both half-life and turnover. Therefore, the purity of labeled amino acids in haemolymph was estimated following thin layer chromatography.

Incorporation of Amino Acids into Haemolymph Soluble Protein

Incorporation of amino acids into haemolymph soluble protein occurs at variable rates (Table 3) depending upon stage in development and amino acid titer. Decreases in the rates of incorporation were exhibited in the transition between the feeding fifth larval stage and the PS or PS+1 days stages of development. Lysine remained fairly constant at about 0.25-0.30 micromoles/hr-animal throughout the LPT, whereas proline and glutamate dropped to very low levels (0.01-0.10 micromoles/hr.-animal) through PS+2 days before increasing 30 and 5 fold respectively (0.3 and 0.5 micromoles/hr-animal) at PS+4 days. Incorporation of leucine, valine and arginine into protein after the feeding larval stage remained depressed throughout the larval-pupal transformation.

Table 2. Rates of turnover of selected amino acids in haemolymph of M. sexta throughout t e LPT. Values are expressed as micromoles hr^{-1} $animal^{-1}$ as alculated from Equation 5.

	Feeding Fifth Instar	Pink Stripe	Stripe +1 Day	Pink Stripe +2 Days	Pink Stripe +3 Days	PinkPink Stripe +4 Days
Arginine	.35	.69	.26	.51	.45	.11
Glutamate	.34	.06	.06	.05	.57	1.35
Histidine	1.35	.12	1.54	.89	.63	.71
Leucine	.86	.59	.35	.38	1.08	.46
Lysine	1.42	.78	.55	.37	.55	.44
Proline	3.07	.64	.61	.86	2.00	1.77
Valine	1.32	1.78	.42	.49	1.69	.79

Table 3. Incorporation of amino acids into haemolymph soluble protein in M. sexta throughout the larval-pupal transformation. Values are expresses as micromoles hr^{-1} $animal^{-1}$ as calculated using Equation 2.

	Feeding Fifth Instar	Pink Stripe	Pink Stripe +1 Day	Pink Stripe +2 Days	Pink Stripe +3 Days	Pink Stripe +4 Days
Arginine	.29	.34	.16	.19	.09	.12
Glutamate	.09	.01	.01	.03	.24	.54
Histidine	.85	.09	.69	.44	.18	.21
Leucine	.32	.35	.10	.11	.21	.15
Lysine	.38	.19	.28	.22	.26	.29
Proline	.19	.06	.08	.11	.24	.33
Valine	.32	.13	.08	.08	.08	.13

IV. DISCUSSION

The nature and roles of the high concentrations of amino acids in haemoplasm and cells of insects have been the subject of numerous studies and reviews (see citations in Hackman, 1956; Duchateau and Florkin, 1958; Wyatt, 1961; Begg, 1962; Ganti, 1963; Schaeffer, 1964; Coles, 1965; Pickett, 1966; Chaput and Liles, 1969; Stidham and Liles, 1969; Uhler et al., 1971; Leader, 1972; Ikan, 1973; Barrett, 1974; Barrett and Lai-fook, 1974; Florkin and Jeuniaux, 1974; Collett, 1976; Firling, 1977; Jungreis, 1978; Staddon and Everton, 1979; Irving, Wilson and Osborne, 1979; Jungreis, 1980). In these studies, stage, diet and species specific concentrations of selected free amino acids were seemingly shown to remain constant. Since members of different orders of insects and even within orders display a wide range of concentrations, these observations of presumptive constancy were then employed to validate haemolymph free amino acids as taxonomic tools for classifying insects and other arthropods (Micks and Gibson, 1957; Sutcliffe, 1963). For example, representative species in primitive orders such as Odonata and Orthoptera have quantities of amino acids in haemolymph that contribute as little as 8% of the total osmotic equivalents, while in more advanced orders such as Lepidoptera, Diptera and Coleoptera, this contribution often reaches 40% (see Duchateau and Florkin, 1958). Although fates of amino acids and their levels in haemolymph are associated with various physiological functions: e.g., the use of proline and/or alanine as major energy substrates during flight—Hargrove, 1975; Brouwers and Dekort, 1978; Weeda and Dekort, 1979; Mordue and Dekort, 1977; as indicators of metabolic scope—Schaeffer, 1964; Pickett and Friend, 1966; Rock and Hodgson, 1970; as determinants in the regulation of haemolymph osmolality--Begg and Cruickshank, 1962; Leader and Bedford, 1972; Chamberlin

and Phillips, 1980; Woodring and Blakeney, 1980; as precursors in cuticle formation—Levenbook, 1969; Anderson, 1977, Koeppe and Gilbert, 1974; and as precursors in fibroin synthesis prior to and during spinning in Bombyx—see citations in Florkin and Jeuniaux, 1974, the wide range of concentrations between species and amongst stages within individual species has made it difficult to make general statements about the roles of free amino acids in insects.

The apparent constancy of amino acids in a species can be demonstrated only with reference to particular stages of development. Extensive research on whole body and haemolymph amino acid levels during development and aging has revealed dramatic and seemingly independent changes of most free amino acid levels. Characteristic stage and species specific patterns of haemolymph (and cellular) amino acids have been determined in only five insect orders: a) Diptera, as exemplified by Drosophila and Culex (Chen, 1960), Aedes aegypti (Micks and Ellis, 1952; Stidham and Liles, 1969 a and b; Chaput and Liles, 1969), Corcyra cephalonica (Gant and Shanmugasandaram, 1963) Phormia regina (Levenbook, 1966), Calliphora (Hackman, 1956; Irving, Wilson and Osborne, 1979), Chironomus tentans (Firling, 1976), b) Lepidoptera, Bombyx mori (Florkin, 1959; Wyatt, et al., 1956), Galleria mellonella (Auclair and Dubreuil, 1952), Macrothylacia rubi (Drilhon, 1952), Manduca sexta (Jungreis, 1980), c) Coleoptera, Leptinotarsa decimlineata (Dekort and Kramer, 1976), d) Hemiptera, Rhodnius prolixus (Barrett, 1973) and e) Orthoptera, Melanoplus differentialis (Fu, 1957). The stage specific constancy of amino acids reflects an overall balance between storage, metabolism and excretion at these stages and leads one to project extensive homeostatic control.

The consistent presence of stage specific concentrations of free amino acids in haemolymph of M. sexta argues for tight rather than loose homeostatic control. Aspects of this regulation in various insects have been elicited through various types of experiments:

1. Changes in diets (including starvation) whose amino acid availabilities differed, followed either by an analysis of haemolymph amino acid concentration changes and/or analysis of limitations to proper development (Bursell, 1963; Strong, 1964; Kasting and McGinnis, 1966; Rock and King, 1968; Rock and Hodgson, 1971; Bosquet, 1977; Woodring and Blakeney, 1980).

2. Administration of radioactively labelled amino acid(s) or peptides to animals, followed by analysis of any or all tissues for label, as well as analysis of excreta and/or respiratory products (Dinamarca and Levenbook, 1966, Stidham and Liles, 1969; Langley and Pimley, 1974; Moloo, 1977; Collett, 1976; Irving, Osborne and Wilson, 1979).

3. Administration of labelled precursors of amino acids and determination of incorporation into various amino acids to determine physiological capacities (Moloo, Langley and Balogun, 1974; Irving, Osborne and Wilson, 1979).

4. Injection of labelled amino acid(s) into haemolymph to determine a rate of turnover (Collett, 1976; Irving, Osborne and Wilson, 1979).

When free amino acid pools are examined using method four, as was done in this study on M. sexta, the nature of the homeostatic regulation is revealed. Pioneer researchers in insect physiology, struck by the high free amino acid levels in comparison to mammalian systems, interpreted large haemolymph pool sizes and concentration as evidence of a static sink function, where changes in concentrations of specific amino acids indicated a changing tissue utilization. While this may indeed be true, little attention has been devoted to measuring

the actual rates either of amino acids exchange between hemolymph and tissues, or the turnover of haemolymph amino acid pools. Turnover experiments can powerfully and clearly define the dynamic quality of a system therein permitting basic questions to be answered and others approached with considerable insight.

In studies on amino acid turnover in Calliphora by Collett (1976), and turnover of alanine and lysine in Phormia (Levenbook and Dinamarca, 1966), dissappearance of label from hemolymph predictably appeared to follow first-order kinetics. Equations generated form a series of linear regression analyses of log transformed data in this study support these earlier observations, with correlation coefficient, r^2, values ranging from .85 to .95, indicating a good fit of data to the generated decay equation. Regression equations for those amino acids with longer half-lives had the lower correlation values (histidine) of about .85. This is due to a smaller change in detected radioactivity among collection points, with a resultingly smaller among sums of squares, coupled with 'constant' within sums of squares. Circumstance makes biological variation (within error) more of a factor.

Incorporation of amino acids into haemolymph soluble protein is recorded only at 3 hours after administration of label. These values give relative rather than absolute rates of synthesis and appearance of selected proteins into haemolymph. No apparent relationship exists between concentration and/or quatity of amino acid in haemolymph and appearance of specific labels into haemolymph proteins. Since account is not made of amino acid incorporation into proteins synthesized and retained by the tissues, all calculations are underestimates of actual rates of incorporation. That degradation of newly synthesized protein could rapidly follow incorporation is another cause of underestimation.

A number of conclusions can be drawn from this study. Firstly, the original premise that amino acids are stored in haemolymph is confirmed for histidine, but this amino acid is clearly an exception to the dynamically maintained amino acid constituents in haemolymph. Secondly, amino acids in haemolymph are regulated, as evidenced by the reproducible stage specific concentrations and half-lives. Although by comparison with mammalian systems, this regulation is not as tight, it is clearly a major determinant of insect homeostasis. The special roles of certain amino acids, such as histidine in osmotic regulation, proline in energy production, and glutamate in neuromuscular activity, could justify the large quantities and concentrations present in Manduca haemolymph. From the apparent constancy of histidine in haemolymph, coupled with the reproducibility of stage specific concentrations of other amino acids, one can infer that changes in blood volumes during development are active and compensated, thereby supporting the concept of active regulation. Lastly, studies on amino acid-incorporation into haemolymph protein demonstrate a rapid exchange of amino acids between cells and haemolymph. Since these exchanges appear to be independent of the quantites of amino acids present in haemolymph, mechanisms of amino acid uptake into cells are probably energy dependent or at least mediated.

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V. EPILOGUE

High amino acid levels found in insects have intuitively intrigued insect physiologists, yet after nearly half a century, all that has been gleaned consists of a wealth of data on their concentrations in haemolymph and/or haemoplasm. In virtually all of these studies, amino acid levels were measured with reference to a fixed time (i.e. a single collection), little account being taken of the actual kinetic properties of the various amino acid pools within haemolymph. In this light, it is not surprising that dramatic fluctuations of amino acid levels in response to environmental changes were interpreted as evidence both that amino acid pools are not regulated, and that amino acids are passively stored in haemolymph and utilized according to tissue needs. Unlike vertebrates, the open circulatory system in insects places blood in direct contact with body tissues, thereby permitting free exchange of nutrients and waste products between blood and cells. The present study on turnover of haemolymph amino acids in the tobacco hornworm, Manduca sexta, reveals exchanges that are both rapid and dynamic, thus dispelling the notion that haemolymph pools are maintained statically. Fluctuations in the respective titers of amino acids during development clearly reflect changing tissue requirements. Regardless, the steady state concentrations of these amino acids are regulated, particularly in response to changes in haemolymph osmotic pressure. For example, histidine serves as a major (i.e. more than 5%) osmotic contributor in the haemolymph of most insects and has a half-life from 50 to 150 hours in M. sexta. In contrast, alanine, which like histidine is present at relatively constant levels throughout larval-pupal development, is not only not stored in haemolymph, but to the contrary is rapidly turned over with a half-life of less than 2 hours. In addition to direct incorporation into protein, alanine can be converted to

pyruvate following deamination, which in turn can then either enter the citric acid cycle or upon oxidation to acetate can be utilized in fat synthesis. Although not empirically demonstrated, at the pink strip stage proline is most likely utilized as a substrate for energy production, whereas during the last two days of the larval-pupal transformation, high rates of proline turnover probably reflect incorporation into cuticular protein. Glutamate, which also displays a short half-life and wide fluctuations in concentration, plays an important role in neuromuscular regulation in conjunction with gamma-aminobutryric acid (Jungreis and Omilianowski, 1980). Although no special roles can be assigned with confidence, other amino acids displayed relatively short half-lives, in the range of 5-10 hours, again indicating dynamic regulation.

LIST OF REFERENCES

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Anderson, S. O. (1977) Arthropod cuticles: Their composition, properties, and functions. Symp. Zool. Soc. London 39:7-32.

Auclair, J. L. (1959) Amino acid oxidase activity in the fat body and malphigian tubes of some insects. J. Insect Physiol. 3:57-62.

Auclair, J. L. and Dubreuil, R. (1952) A simple ultramicro method for the quantitative estimation of amino acids by paper chromatography. Can. J. Zool. 30:109-113.

Barrett, F. M. (1974) Changes in the concentration of free amino acids in the hemolymph of Rhodnius prolixus during the fifth instar. Comp. Biochem. Physiol. 48B:241-250.

Barrett, F. M. and Lai-Fook, J. (1974) Free amino acids in the hemolymph of Calpodesethlius during the fifth instar. Comp. Biochem. Physiol 53B:205-210.

Baumhover, A. H., Cantello, W. W., Hosgod, J. M., Knott, C. M., and Lam, J. J. (1977) An improved method for mass rearing the tobacco hornworm. Agricultural Research Service, United States Dept. of Agriculture Publication #ARS-5-167.

Begg, M. and Cruickshank, W. J. (1962) A partial analysis of Drosophila larval haemolymph. Proc. R. Soc. (Edin.) 68:215-236.

Bell, R. A. and Joachim, F. G. (1976) Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. Ann. Ent. Soc. Am. 69:365-373.

Bodnaryk, R. P. and Levenbook, L. (1969) The role of B-alanyl-L-tyrosine (Sarcophagine) in puparium formation in the fleshfly, Sarcophaga bullata. Comp. Biochem. Physiol. 30:909-921.

Bosquet, G. (1977) Hemolymph modifications during starvation in Philosamia cynthia walkeri (Ferber) — II. Amino acids and peptides. Comp. Biochem. Physiol. 58A:377-382.

Brouwers, E. V. M. and Dekort, C. A. D. (1979) Amino acid metabolism during flight in the Colorado potato beetle, Leptinotarsa decimlineata. J. Insect Physiol. 25:411-414.

Bursell, E. (1963) Aspects of the metabolism of amino acids in the tsetse fly, Glossina (Diptera). J. Insect Physiol. 9:439-452.

Chamberlin, M. E. and Phillips, J. E. (1980) Regulation of free amino acids in the desert locust. Abstract. Fed. Proc. 38:907.

Chaput, R. L. and Liles, J. N. (1969) Free and peptide-bound amino acids during the larval and pupal stages of the yellow-fever mosquito, Aedes aegxpti. Ann. Ent. Soc. Am. 62:742-747.

Chen, P. S. (1960) Changes in amino acids and proteins during larval development of Drosophila and Culex. Verh. XI int. Kongr. Ent. 3:201-207.

Chen, P. S. and Bachmann-Diem, C. (1965) Studies on the transamination reactions in the larval fat body of Drosophila melanogaster. J. Insect Physiol. 10:819-829.

Coles, G. C. (1965) The haemolymph and moulting in Rhodnius prolixus Stal. J. Insect Physiol. 11:1317-1323.

Collett, J. I. (1976a) The constancy and similarity of the amounts of free amino acids in inbred strains of Drosophila and Calliphora. J. Insect Physiol. 22:1251-1255.

Collett, J. I. (1976b) Some features of the regulation of the free amino acids in adult Calliphora erythrocephala. J. Insect Physiol. 22:1395-1403.

Collett, J. I. (1976c) Small peptides, a life-long store of amino acids in adult Drosphila and Calliphora. J. Insect Physiol. 22:1433-1440.

Dekort, C. A. D. and Kramer, S. J. (1976) Age related changes in amino acid composition and turnover in the Colorado potato beetle, Leptinotarsa decimlineata Say. Insect Biochem. 6:339-343.

Desai, R. and Kilby, B. A. (1958) Some aspects of nitrogen metabolism in the fat body of the larva of Calliphora erythracephala. Arch. int. Physiol. Biochem. 66:248-259.

Dinamarca, M. L. and Levenbook, L. (1966) Studies on the hemolymph proteins of the blowfly, Phormia regina -I. Changes in ontogenic patterns. J. Insect Physiol. 12:1595-1609.

Duchateau, G. and Florkin, M. (1958) A survey of amino-acidemias with special reference to the high concentration of free amino acids in insect haemolymph. Arch. int. Physiol. Biochem. 66:573-591.

Firling, C. E. (1977) Amino acid and protein changes in the haemolymph of developing fourth instar of Chrionomus tentans. J. Insect Physiol. 23:17-22.

Florkin, M. (1959) The free amino acids of insect haemolymph. Proc. VI int. Cong Biochem. 12:63-73.

Florkin, M. and Jeuniaux, C. (1974) Haemolymph composition. In The Physiology of Insecta (Ed. by Rockstein, M.) 5:255-307. Academic Press, New York.

Fu, Y. Y. (1957) Changes in distribution of sulfur-containing amino acids in grasshopper egg (Melanoplus differentialis). Physiol. Zool. 30:1-12.

Ganti, Y. and Shanmugasundaram, E. R. B. (1963) A study on the free amino acids during growth and metamorphosis of Corcyra cephalonica St. J. Exp. Zool. 152:1-4.

Hackman, R. H. (1956) Changes in the free amino acids of the blood of blowfly larvae at metamorphosis. Aust. J. Biol. Sci. 9:400-405.

Haer, F. C. (1969) An Introduction to Chromatography on Impregnated Fiber. Ann-Arbor:Humphrey Science Publishers. Ann Arbor, MI.

Hargrove, J. W. (1976) Amino acid metabolism during flight in tsetse flies. J. Insect Physiol. 22:309-313.

Ikan, R. and Ishay, J. (1973) Free amino acids in the hemolymph and the venom of the oriental hornet, Vespa orientalis. Comp. Biochem. Physiol. 44B:949-952.

Irving, S. N., Osborne, M. P. and Wilson, R. G. (1979) Studies on L-glutamate in insect haemolymph. I. Effects of injected L-glutamate. Physiol. Entom. 4, 139-146.

Irving, S.N., Wilson, R.G. and Osborne, M.P. (1979) Studies on L-glutamate in insect haemolymph. II. Distribution and metabolism of radiolabelled amino acids injected into the haemolymph. Physiol. Entom. 4:223-230.

Irving, S. N., Wilson, R. G. and Osborne, M. P. (1979) Studies on L-glutamate in insect haemolymph. III. Amino acid analyses of the haemolymph of various arthropods. Physiol. Entom. 4, 231-240.

Jenkin, P. M. and Hinton, H. E. (1966) Apolysis in arthropod moulting cycles. Nature, Lond. 211:871.

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. (1979) The physiology of moulting in insects. In Adv. Insect Physiol. 14:109-183.

Jungreis, A. M. (1980) Hemolymph as a dynamic tissue. In Insect Biology in the Future (Ed. by Locke, M. and Smith, D. S.) pp. 273-294, Academic Press, New York.

Jungreis, A. M. and Omilianowski, D. R. (1980) -Amino butyric acid and glutamic acid in Manduca sexta: Proposed roles in insect development. In press, Comp. Biochem. Physiol. 67C, 174-185.

Kasting, R. and McGinnis, A. J. (1966) Radioisotopes and the determination of nutrient requirements. Ann. N.Y. Acad. Sci. 139:98-107.

Koeppe, J. K. and Gilbert, L. I. (1974) Metabolism and protein transport of a possible pupal cuticle tanning agent in Manduca sexta. J. Insect Physiol. 20:981-992.

Langley, P. A. and Pimley, R. W. (1974) Utilization of U-^{14}C amino acids or U-^{14}C proteins by adult Glossina moristans during In Utero development of larva. J. Insect Physiol. 20:2157-2170.

Leader, J. P. and Bedford, J. J. (1972) The composition of the New Zealand earwig, Anisolabis littorea. J. Insect Physiol. 18:2229-2235.

Lee, P. L. (1974) Single column system for accelerated amino acid analysis of physiological fluids using five lithium buffers. Biochem. Med. 10:107-121.

Levenbook, L. (1966) Hemolymph amino acids and peptides during larval growth of the blowfly, Phormia regina. Comp. Biochem. Physiol. 18:341-351.

Levenbook, L. and Dinamarca, M. L. (1966) Free amino acids and related compounds during metamorphosis of the blowfly, Phormia regina. J. Insect Physiol. 12:1343-1362.

Levenbook, L. and Dinamarca, M. L. (1966) Oxidation, utilization, and incorporation into protein of alanine and lysine during metamorphosis of the blowfly, Phormia regina (Meigen). Arch. Biochem. Biophys. 117:110-119.

Levenbook, L. R., Hutchins, F. N. and Bauer, A. C. (1971) Uric acid and basic amino acids during metamorphosis of the tobacco hornworm, Manduca sexta, with special reference to the meconium. J. Insect Physiol. 17, 1321-1331.

Levenbook, L. and Krishna, I. (1971) Effect of aging on amino acid turnover and rate of protein synthesis in the blowfly, Phormia regina. J. Insect Physiol. 17:9-12.

Micks, D. and Ellis, J. P. (1952) Free amino acids in developmental stages of the mosquito. Proc. Soc. exp. Biol. Med. 79:191-193.

Micks, D. W. and Gibson, F. (1957) The characterization of insects and ticks by their free amino acid patterns. Ann. Ent. Soc. Am. 50:500-505.

Moloo, S. K. (1977) Aspects of the metabolism of U-^{14}C arginine, U-^{14}C histidine and U-^{14}C lysine by adult female Glossina moristans during pregnancy. Comp. Biochem. Physiol. 57B:23-26.

Moloo, S. K., Langley, P. A. and Balogun, R. A. (1974) Amino acid synthesis from U-^{14}C glucose in Glossina moristans. J. Insect Physiol. 20:1807-1813.

Mordue, W. and Dekort, C. A. D. (1978) Energy substrates for flight in the Colorado potato beetle, Leptinutarsa decimlineata Say. J. Insect Physiol. 24:221-224.

Nijhout, H. F. and Williams, C. M. (1974) Control of moulting and metamorphosis in the tobacco hornworm, Manduca sexta (L.): Growth of the last instar larva and the decision to pupate. J. Exp. Biol. 61:481-491.

Patton, R. L. (1963) Introductory Insect Physiology p. 48. W. B. Saunders, Philadelphia.

Pickett, C. and Friend, W. G. (1966) Free amino acids in the hemolymph of Rhodnius prolixus Stal (Hemiptera: Reduviidae). Can. J. Zool. 44:241-249.

Price, G. M. (1961) Some aspects of amino acid metabolism in the adult housefly, Musca domestica. Biochem. J. 80:420-428.

Riddiford, L. M., Kiguchi, K. Roseland, C. R., Chen, A. C. and Wolfgang, W.J. (1980) Cuticle formation and sclerotization in vitro by the epidermis of the tobacco hornworm, Manduca sexta. In Invertebrate Systems In Vitro (Ed. by Kurstak, C., Maramorsch, W., Dubendorfer, D.). North-Holland Biomedical Press, Elsevier, Neth.

Rock, G. C. and King, K. W. (1968) Amino acid synthesis from glucose-U-^{14}C in Argyrotaenia velutinana (Lepidoptera: Tortricidae) larvae. J. Nutr. 95:369-373.

Rock, G. C. and Hodgson, E. (1971) Dietary amino acid requirements for Heliothis zea determined by dietary deletion and radiometric techniques. J. Insect Physiol. 17:1087-1097.

Rohlf, F. J. and Sokal, R. R. (1969) Statistical Tables. W. H. Freeman and Co., San Francisco.

Schaeffer, C. H. (1964) Free amino acids of the Virginia pine sawfly, Neodiprian pratti Dyar: their chromatographic determination and biosynthesis. J. Insect Physiol. 10:363-369.

Sokal, R. R. and Rohlf, F. J. (1969) Biometry. W. H. Freeman and Co., San Francisco.

Staddon, B. W. and Everton, I. J. (1979) Hemolymph of the milkweed bug Oncopeltus fasciatus (Heteroptera; Lygaeidae): Inorganic constituents and amino acids. Comp. Biochem. Physiol. 65A:371-374.

Stidham, J. D. and Liles, J. N. (1969) Free amino acid composition of the aging female mosquito, Aedes aegypti, as determined by automatic ion-exchange chromotography. J. Insect Physiol. 15:1969-1980.

Stidham, J. D. and Liles, J. N. (1969) Some aspects of the metabolic fate of ^{14}C-labeled alanine and aspartic acid in the aging female mosquito, Aedes aegypti (L.) (Diptera:Culicidae). Comp. Biochem. Physiol. 31:513-521.

Strong, F. E. (1964) The effects of nitrogen starvation on the concentration of free amino acids in Myzus persicae (Sulzer) (Homoptera; aphidae). J. Insect Physiol. 10:519-523.

Sutcliffe, D. W. (1963) The chemical composition of haemolymph in insects and some other arthropods in relation to their phylogeny. Comp. Biochem. Physiol. 9:121-135.

Taylor, D. P. and Newbergh, R. W. (1979) The synthesis and content of neurotransmitters and their effect on cyclic nucleotide accumulation in the central nervous system of Manduca sexta. Insect Biochem. 9:265-272.

Uhler, L. D., Crispen, C. R., and McCormick, D. B. (1971) Free amino acid patterns during development of Eurosta solidagnis Fitch. Comp. Biochem. Physiol. 38B:87-91.

Weeda, E. and DeKort, C. A. D. (1979) Fuels for energy metabolism in the Colorado potato beetle, Leptinotarsa decimlineata (Say). J. Insect Physiol. 25:951-955.

Whitehead, D. L. (1969) New evidence for the control mechanism of sclerotization in insects. Nature, Lond. 224:721-723.

Wigglesworth, V. B. (1965) The Principles of Insect Physiology, 6th Edition. Methuen, London.

Williams-Boyce, P. K. and Jungreis, A. M. (1980) The larval-pupal transformation of Manduca sexta: Changes in body tissue weights and amount of haemolymph contaminating the tissues. Ann. Ent. Soc. Am. 70, 602-607.

Woodring, J. P. and Blakeney, E. W. (1980) The role of free amino acids in osmoregulation of cricket blood (Acheta domesticus). J. Insect Physiol. 26:613-618.

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

APPENDIX

Table A-1. Abbreviated staging criteria for diet reared (24-25°C) Manduca sexta before, during and after the larval-pupal transformation. Detailed descriptions of staging criteria have been summarized in Jungreis (1979).

Stage	Time	Characteristics
feeding fifth instar	1 day before pink stripe	7.5-10 g turquoise-blue larva; initiation of wandering
pink stripe	5 days before larval-pupal ecdysis	dorsal aorta visible; pink stripe (ommochrome pigment) along dorsum
Day 1	4 days before larval-pupal ecdysis	animal continues to be motile; disperal of ommochrome pigment; initiation of ocellar retraction.
Day 2	3 days before larval-pupal ecdysis	animal bends or twists, but is not motile; ocellar retraction complete.
Day 3	2 days before larval-pupal ecdysis	clear ecdysial line on dorsum of thorax; animal unable to bend or twist.
Day 4	1 day before larval-pupal ecdysis	dorsal patches of pigment on metathorax on both sides of ecdysial line
Day 5	day of larval-pupal ecdysis	ecdysis of larval cuticle; thorax light green becoming light brown; proboscis and wing sacs extended; abdomen more deeply tanned than thorax.

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