To the Graduate Council:

I am submitting herewith a thesis written by Patsy Kylene Williams Boyce entitled "Regulation of Fat Body Urate Accumulation in the Development of the Tobacco Hornworm, Manduca sexta." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Zoology.

Arthur M. Jungreis, Major Professor

We have read this thesis
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REGULATION OF FAT BODY URATE ACCUMULATION IN THE DEVELOPMENT OF THE TOBACCO HORNWORM, *MANDUCA SEXTA*

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Patsy Kylene Williams Boyce

December 1977

1345640

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DEDICATION

Regulation of Fat Body Urate Accumulation in the Development of the Tobacco Hornworm, Manduca sexta is dedicated to my mother, Etoliah D.W. Caldwell, whose love for animals is unbounded. From her example of love I learned that each living creature adds something very vital to our world, whether it be in the screech of a barn owl, the bellow of a cow, or the busy scamperings of a dung beetle. She always accepted the creatures I dragged home, from the junk-pile kitten, and water-logged field mouse to the hay loft spiders. Most admirable are her dedication to life even in its grimmest moments, her seeing of good in every person, and, in times when there were not material things to share, her giving those around her extra helpings of love. I would like to honor her with this small token of my love and appreciation.

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ABSTRACT

The regulation of fat body uric acid accumulation was studied during the larval-pupal transformation of the tobacco hornworm, Manduca sexta.

Uric acid, the primary nitrogen waste product synthesized and eliminated by M. sexta is not retained by individual tissues prior to the larval-pupal transformation (LPT). Onset of the LPT heralds an increase in urate storage (17 → 136 μmoles/animal), with accumulation occurring primarily in fat body (9 → 113 μmoles/animal). Despite the increase in accumulation, fat cell urate synthesis in vitro declined 77% during the LPT. Ligation of pink stripe pupae (PS) revealed hormonal control of urate synthesizing capacity. Posterior sections exhibited a 3 fold increase in retained urate synthesizing capacity relative to anterior sections on the day of pupation. Paired ratios from ecdysone-treated ligates are identical to untreated ligates. Beginning on successive days of the LPT and measured on the day of ecdysis, involvement of ecdysone in the control of urate synthesis was studied by daily hormone administration to non-ligated animals. Ecdysone prevented the loss in urate synthesizing capacity until 36 hours before ecdysis, urate deposition continuing at the "normal" rate. It is proposed that fat body urate storage is regulated during the LPT by precursor availability and not by the changes in urate synthesizing capacity elicited by ecdysone.

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INTRODUCTION

The basic concepts relating phylogeny of the patterns of nitrogen excretion were developed in the 1930's by Delaunay (1931), Needham (1933) and Baldwin (1937). Excretion of nitrogenous waste products by insects has been reviewed by Wigglesworth (1950), Prosser (1952), Craig (1960, Corrigan (1970) and Stobbart and Shaw (1974). In these treatises, the subjects of purine metabolism and nitrogen excretion are inextricably intertwined. The earlier interpretation that all (see citations in Patton, 1953) terrestrial insects are uricotelic (i.e. exreting uric acid as their main nitrogenous waste product) has been modified by the work of Manuta (1948, 1949) and Razet (1953, 1956, 1961, 1966). Razet examined 426 species at different stages in development and noted that while some insects excrete primarily uric acid, others excrete in addition to urate allantoin, allantoic acid, and urea.

The capacity to synthesize and excrete uric acid is characteristic of more highly evolved organisms (Balinsky, et al., 1976). The majority of insects are terrestrial, thus their evolution has dictated uricotelism or purinotelism as their main modes of excretion with a concomitant alteration (increase) in enzymes leading to the production of uric acid.

The capacity to store excretory nitrogen is possessed by many organisms. This waste nitrogen is retained by one or more tissues until conditions permit its elinination (Mullins and Cochran, 1976). Internal sotrage of uric acid occurs in organisms of diverse taxonomic

groups such as Arthropoda: Class Insecta (Haydak, 1953; McEnroe, 1957; Maddrell, 1971; Cochran, 1973, 1975), Class Isopoda (Hartenstein, 1968), Class Crustacea (Horne, 1968), Mollusca: Class Gastropoda (Badman, 1971), Chordata: Class Ascidiacia (Nolfi, 1970).

In holometabolous (i.e.,metamorphosis including larva, pupa, adult) insects, metamorphic changes occurring between the feeding larval and adult stages (i.e.,the pupa being a closed system) in development exacerbate the problems of waste removal and necessitate considerable selective storage of a wide variety of materials including uric acid. The primary nitrogen waste product synthesized and eliminated by the tobacco hornworm, Manduca sexta, is uric acid. Little uric acid is retained by feeding fifth instar larvae, but considerable uric acid is found in hornworms that have just completed the larval-pupal ecdysis (Levenbook,et al., 1971). The control mechanisms involved in the production, storage, and excretion of uric acid in this or in other species have not been determined.

Production of uric acid in relation to protein metabolism in the silkworm, Bombyx mori was studied by Tojo (1971) during pupal-adult development. In this species, nitrogen lost from protein and free amino-acids could be correlated with the increase in urate nitrogen. Tojo showed that at certain stages in pupal and adult development, uric acid is produced from "surplus" protein derived from the resorbed tissues. These studies left several questions unanswered. What kind of regulatory mechanisms are operative during the rapid production of uric acid? Which tissues are responsible for the production of uric acid? Which tissues store uric acid? Is uric acid synthesized in certain tissues and stored in other tissues?

In M. sexta the transition from larva to adult has been studied exhaustively and found to be under strict hormonal control, (King, 1972; Truman and Riddiford, 1974; Bollenbacher, et al., 1975; Sridhara and Gilbert, 1975; Riddiford, 1976 a,b; Nijhout, 1976; Mitsui and Riddiford, 1976). It seems logical then that the metabolic processes accompanying this metamorphosis, such as the selective synthesis and storage of uric acid, might also be hormonally controlled.

The present study examines some of the questions concerning the control of uric acid accumulation during the larval-pupal transformation, namely: (1) What is the normal pattern of urate acculumation? (2) Can this tissue or these tissues synthesize urate at a rate sufficient to account for its accumulation there? (3) How is this rate of synthesis regulated? and (4) If synthesis is hormonally controlled, at what proposed level of synthesis does the hormone act: a) through enzyme activation-inactivation; b) through stimulation of enzyme production or degradation; c) or through precursor availability?

CHAPTER I

MORPHOLOGICAL OBSERVATIONS DURING THE LARVAL-PUPAL TRANSFORMATION

Introduction

The tobacco hornworm, Manduca sexta L is being used by insect physiologists with increasing frequency as a model for developmental events in Lepidopterous insects. Unfortunately, most investigators have found under a variety of well-defined conditions of photoperiod, temperature, humidity and diet that both the weight of individuals as well as the duration of developmental stages cannot be defined with a high degree of precision (Bell and Joachim, 1976; Lockshin, et al., 1975; Truman and Riddiford, 1974; Dahlman, 1973 a,b; Nijhout and Williams, 1974). Hornworms, as holometabolic insects, exhibit metamorphoses which necessitate complex and prolonged stage-specific transformations. Throughout the larval-pupal transformation, pharate pupae are characterized by marked changes in weight and hormone sensitivity (Nijhout and Williams, 1974; Fain and Riddiford, 1975; Riddiford, 1976). While populations of animals can be classified as a function of mean body weights, individuals can be staged only with difficulty. Moreover, the bases of these weight changes are only poorly understood. Therefore, morphological features characteristic of animals throughout the larval-pupal transformation as well as the contribution by major organs to the total body weight were determined. A knowledge of body composition is necessary if tissue from animals of different body weights are to be compared. Finally, true tissue weights were determined by correcting for adhering hemolymph.

Materials and Methods

Animals. Feeding larvae of the tobacco hornworm, Manduca sexta, were individually housed in 8 ounce plastic containers and maintained on a synthetic diet (Bell and Joachim, 1976) under conditions of constant temperature (22-25°C), humidity (70%) and photoperiod (L:D,18:6). Following the onset of wandering (i.e., pink stripe stage) individuals were placed in stoppered wooden blocks (into which holes 2.5 cm in diameter and 17.5 cm in depth had been drilled), to simulate the natural earthen burrow. At various times throughout the larval-pupal transformation, animals were removed and staged within a maximum period of ± 6 hours (see following study) in order to minimize age-related variations in morphology and physiology.

Total Body and Major Tissue Weights. Fifty late fifth instar larvae were weighed on a top loading Mettler balance Model P1200 every 12 hours prior to evacuation of the gut contents, and every 24 hours thereafter until the day of the pupal-adult eclosion and morphological characteristics recorded. Using this information (on changes in weight and morphology during development) a second group of 50 animals was then staged and the major (based on weight) body tissues isolated, blotted on glassine-type weighing paper, and weighed to the nearest tenth of a milligram on a Mettler H30 analytical balance.

Hemolymph Adherence to Tissues. The quantities of hemolymph adhering to the fat body, gut and integumentary tissue, and total hemolymph volume were determined throughout the larval-pupal transformation. Employing carbon dioxide-anesthetized (narcotized) animals, ^{3}H-inulin (633 mCi/μmole, New England Nuclear) was injected from a 10

µl Hamilton microsyringe on the tergum of the prothorax of late feeding fifth instar larvae and prepupae, and behind the eyes in new pupae (2-4 hours after ecdysis) to yield a final concentration of 1000 dpm/µl of hemolymph (initially assuming a hemolymph volume of 3.5 ml or 40% of the total feeding larvae weight and a volume of 1.5 ml or 30% of the body weight in newly ecdysed pupae). Injection sites were then sealed with melted paraffin. Reduced circulation in pupae (see Jones, 1964) necessitated the manual rotation of the abdomen to facilitate more rapid equilibration of inulin in hemolymph between the sampling periods. Animals were re-anesthetized in carbon dioxide at 30,60,90 and 120 minutes following inulin administration. Ten microliter aliquots of hemolymph were collected from the injection site, transferred directly into scintillation vials containing 10 ml of Aquasol (New England Nuclear), and counted in an Amersham Searle Model 6862 Liquid Scintillation Counter. Based on stage-specific equilibration times, inulin-^{3}H was administered to 12 animals on each day of the larval-pupal transformation, (three were feeding fifth larvae, three were -4 stage prepupae, three were -1 stage prepupae and three were newly ecdysed pupae). The tissues were isolated and blotted (phenylthiourea was added to these and all other tissues or fluids to inhibit tyrosinase activity), and approximately 100 mg quantities (weighed on a Roller-Smith torsion balance) were homogenized in 400 µl of 0.05 m Tris-HCl buffer (pH 6.8) in a ground glass tissue homogenizer. Ten and twenty microliter aliquots of homogenate were counted in 10 ml of Aquasol and the percentage hemolymph adherence to the tissues was determined. Actual data

was collected for only 4 of the 7 days of the LPT and the adherence curve was extrapolated from these with the aid of visual observations.

Results

Morphology. Stage-specific morphological features of Manduca sexta during the larval-pupal transformation (LPT) are listed in Table I. The descriptions provided cover features present in both undissected and dissected animals. Changes in internal morphology are limited to gross alterations in tissue size, appearance or content.

Total Body and Major Tissue Weights. Changes in the total body weight of Manduca sexta between the feeding mid-fifth instar larva and the day of adult eclosion are presented in Figure 1. The insert in Figure 1 shows the percentage of the maximum weight (normalized to the average weight of late feeding fifth instar larvae, 9.16 g) occupied by the animals at each day of the LPT, for both 5 (25-27°C) and 6 (20-22°C) day cycles. Total body weights declined 75% during this period (from 9.16 g for the late feeding fifth instar larva to 1.90 g for the eclosed adult) with dramatic losses noted during the LPT (9.16 g - 4.82 g) and on the day of adult eclosion (4.22 g - 1.90 g). The changes in individual tissue weights with the exception of the fat body, reflect this loss in total body weight during the LPT (Table II). Tissue weights have been corrected for adhering hemolymph following equilibration with ^{3}H-inulin. The total volume of hemolymph was determined by difference between the total body weight and the corrected organ weights (values later confirmed by the inulin study) and by assuming that 1 gram of hemolymph will occupy a volume of 1 ml (see Jungreis and Wyatt, 1972). The volume of hemolymph was determined in this manner as a second check

TABLE I

TIMETABLE OF DEVELOPMENT FOR SYNTHETIC DIET REARED TOBACCO HORNWORMS, MANDUCA SEXTA (AT 20-22°C)

Day-Age	Characteristics
Feeding fifth -10 Days Prepupa	Weight 2.5-10 grams. Legs able to hold and grasp Integument dull in appearance, not shiny-wet; No swelling or puffiness noted in prolegs. Cuticle turquoise; Gut distended with food; Fecal pellets more darkly colored than food and uniform in size. Large salivary glands; Fat body diffuse with large amount of adhering hemolymph
Evacuating fifth -6 Days Prepupa	Large fecal pellets indicative of undigested food with a frosted appearance Anterior integument wet in appearance (Njhout and Williams, 1974) Prolegs puffy; Animal firm upon squeezing Gut filled with lightly colored undigested food
Evacuated fifth (Pink Stripe) First 12 hours -5 Days Prepupa	Pink stripe (omnochrome pigment) dorsally with dorsal aorta visible Not feeding: Initiation of wandering; Body firm Gut collapsed
Late Pink Stripe Last 12 hours	Pink Stripe dark Wandering; Negative geotaxis; Very active Body firm; Gut filled with clear gel-like fluid
-4 Days Prepupa	Animal remains motile, though soft; Integument not separated from pupal cuticle Color dissipating from dorsum, color light pink to clear Head and thoracic integument appear yellow-green Prolegs unable to grasp; Legs and mandibles still active Gut filled with clear fluid at early -4 Salivary glands decreasing in size

Table I (Continued)

Day-Age	Characteristics
-3 Days Prepupa	Pigment stripe dissipated; Dorsal aorta clear Entire animal yellow-green Legs non-functional and prolegs retracted. Animal can only bend or twist White ecdysial line appears medially on the dorsum between 3rd and 4th segments Gut empty Small amount of molting gel present; Manual separation of larval from pupal cuticles impossible.
-2 Days Prepupa	Entire animal cigar-shaped; Body firm Fat body visible through abdominal wall Dorsal aorta faint or not visible Prolegs slightly tanned White ecdysial line very prominent Molting fluid present between prominently separated larval and pupal cuticles Salivary glands difficult to dissect free due to small size Fat body increasing in mass; Decrease in adhering hemolymph to fat body Small amount of pale orange gut contents
-1 Prepupa 24-12 hours prior to ecdysis	Animal cigar shaped and body turgid White ecdysial line visible anterior region medially with light prependicular band underneath. Very slight tanning ventrally at last abdominal segments; Spotted cuticle visible underneath; Abdominal horn still prominent Dark brown gut contents Cuticles easily separated Entire animal light yellow-green

Table I (Continued)

Day-Age	Characteristics
14-8 hours prior to ecdysis	Head tilted ventrally approximately 45° to the plane of the body Prependicular band under ecdysial line darker Larval cuticle pliable but not easily torn Abdominal horn worn down Much molting fluid present Dark gut contents
8-4 hours prior to ecdysis	Band on head very dark Abdominal pupal cuticle dark and extremely visible Larval cuticle wrinkled in appearance and easily torn Head tilted perpendicular to body Animal soft Dark gut contents Little molting fluid present
Pupa	Pupa completely tanned abdominally Tanning complete 2-4 hours after ecdysis Proboscis lengthens and wings cover thorax Anterior region light green until tanning occurs Salivary glands not obvious Very little hemolymph present in abdomen Fat body glue-like Dark gut contents

Figure 1. Changes in the body weight of Manduca sexta between the feeding fifth larval instar and the newly eclosed adult. Each value is an average of fifty animals. (The standard errors were less than 0.01% of listed weights.) The solid lines represent larvae reared at 20-22°C (6 day larvae-pupal transformation) while the broken lines represent larvae reared at 25°C. (5 day LPT) Insert: Percent of maximum weight occupied by animals on each day of LPT, assuming a normalized maximum weight of 9.16 g for feeding larvae. FF = feeding fifth instar larvae, PS = Pink Stripe, LPE - day of larval-pupal ecdysis, E = adult eclosion. Numbers correspond to days before or after the larval-pupal ecdysis.

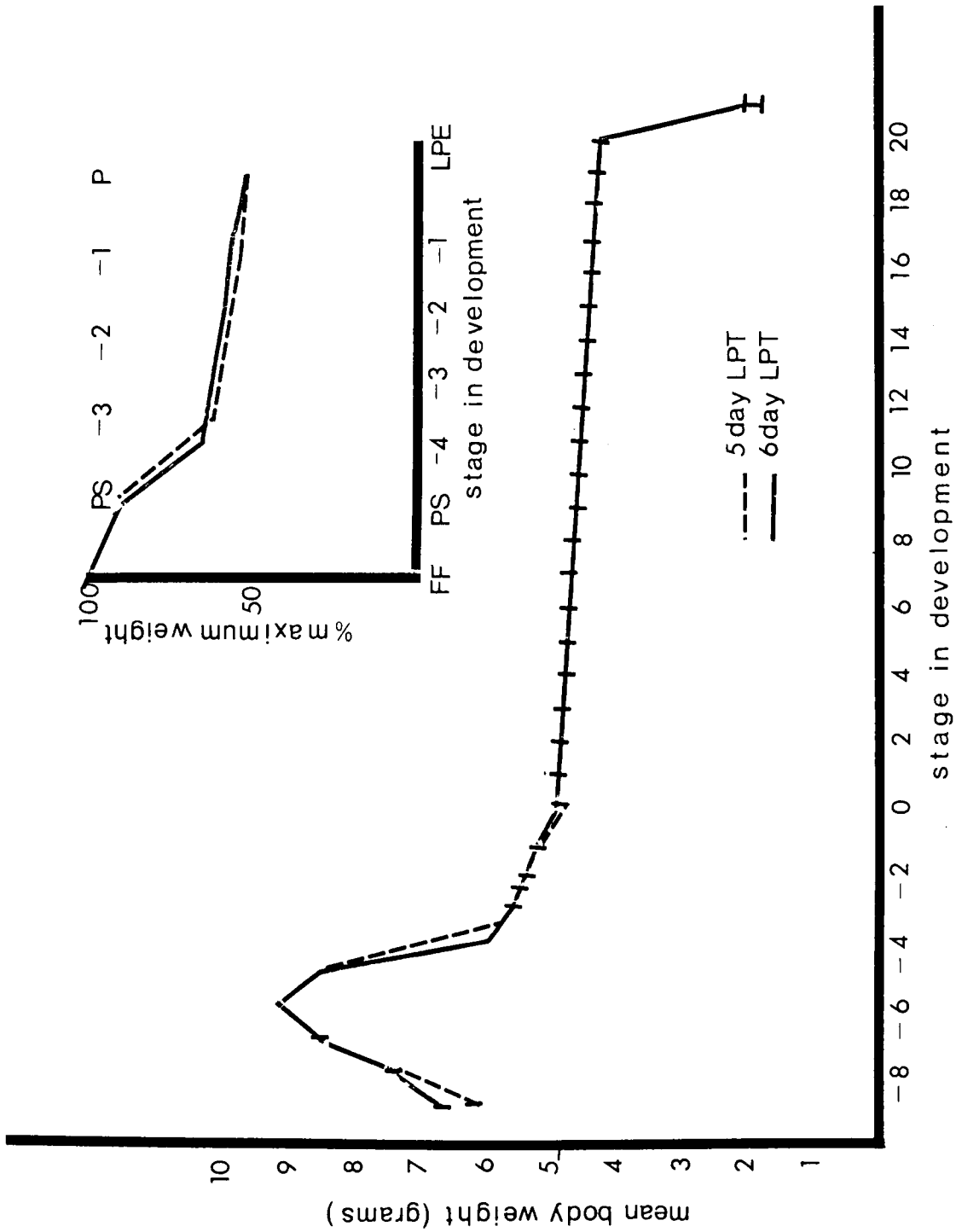
mean body weight (grams)
stage in development
5day LPT
6day LPT
% maximum weight
FF PS -4 -3 -2 -1 LPE
PS -3 -2 -1 P
stage in development

Figure 1

TABLE II

CHANGES IN WEIGHTS OF MAJOR BODY TISSUES DURING THE LARVAL-PUPAL TRANSFORMATION

Stage*	Body (BW)	n**	Gut Contents	%BW	Gut	%BW	Fat Body	%BW	Integument	%BW	Hemolymph	%BW
FF	9.16	9	2.29 ±0.23	25	0.34 ±0.04	4	0.52 ±0.05	6	2.36 ±0.15	26	3.65	40
PS	8.44 ±0.04	6	1.41 ±0.04	17	0.34 ±0.04	4	0.53 ±0.05	6	2.19 ±0.13	26	3.97	47
-4	5.98 ±0.06	5	0.05 ±0.02	1	0.28 ±0.04	5	0.53 ±0.06	9	1.91 ±0.13	32	3.20	54
-3	5.56 ±0.05	4	0.04 ±0.01	1	0.10 ±0.01	2	0.56 ±0.05	10	2.06 ±0.09	37	2.76	50
-2	5.38 ±0.05	9	0.12 ±0.02	2	0.09 ±0.01	2	0.69 ±0.13	1	1.87 ±0.12	35	2.6	48
-1	5.19 ±0.06	4	0.28 ±0.12	5	0.20 ±0.02	4	0.77 ±0.08	15	1.93 ±0.23	37	2.01	39
LPE	4.87 ±0.06	4	0.95 ±0.09	20	0.17 ±0.02	4	0.69 ±0.07	14	1.61 ±0.06	33	1.44	30

Values in grams ± S.E. of the means
*See legend in Figure 1.
**n = Number of separate determinations
***Includes foregut, midgut, hindgut, rectum, malpighian tubules

of accuracy for the inulin study, since the amount of inulin absorption can very with developmental tissue changes (Cherbas and Cherbas, 1970). Variations in body weight, which would affect the absolute tissue weights, but not their percentile contribution to the total weight, were normalized to the mean stage-specific weight and tissue weight corrections were made accordingly. The integument decreases in weight by 32% between the feeding fifth instar larva and the new pupa. The volume of gut contents decreases by 97% between the feeding fifth instar larva and the -3 day prepupa after which it's weight increases some 12 fold by the day of the larval-pupal ecdysis. The gut decreased 50% in weight from 0.34 g at the feeding fifth stage to 0.17 g at the larval-pupal ecdysis, yet, the loss at days -3 and -2 prepupa is 71% indicating a large initial decrease in weight, then an increase. The greatest weight changes are recorded for hemolymph which decreased 60% (3.65 g to 1.44 g) during this period. In contrast to integument, gut, and hemolymph which all experience losses in weight during the LPT, the weight of fat body increases 33% from 0.52 g in the feeding fifth larva to 0.77 g one day before the larval-pupal ecdysis.

Inulin Study. The percentage of hemolymph adhering to fat body, gut and integument during the larval-pupal transformation was determined (Figure 2). Hemolymph was found to occupy 26.5% by weight of the feeding fifth larval fat body, 14% of the integument and 10% of the gut. Hemolymph adherence accounted for 13.5% of the new pupal fat body weight, but less than 2% of the pupal integument and only 5.5% of the pupal gut weight. The volume of hemolymph was found to be at 3.8 - 4.0 ml for the feeding fifth instar and 1.4 - 1.54 ml for the new pupa in agreement with values listed in Table II.

Figure 2. Percentage of hemolymph adherence to the major body tissues as determined by the marker, ^{3}H-inulin during the larval-pupal transformation. Circled points indicate actual data points, other points are extrapolated. FF = feeding fifth instar larvae, PS = pink stripe, LPE = day of larval-pupal ecdysis.

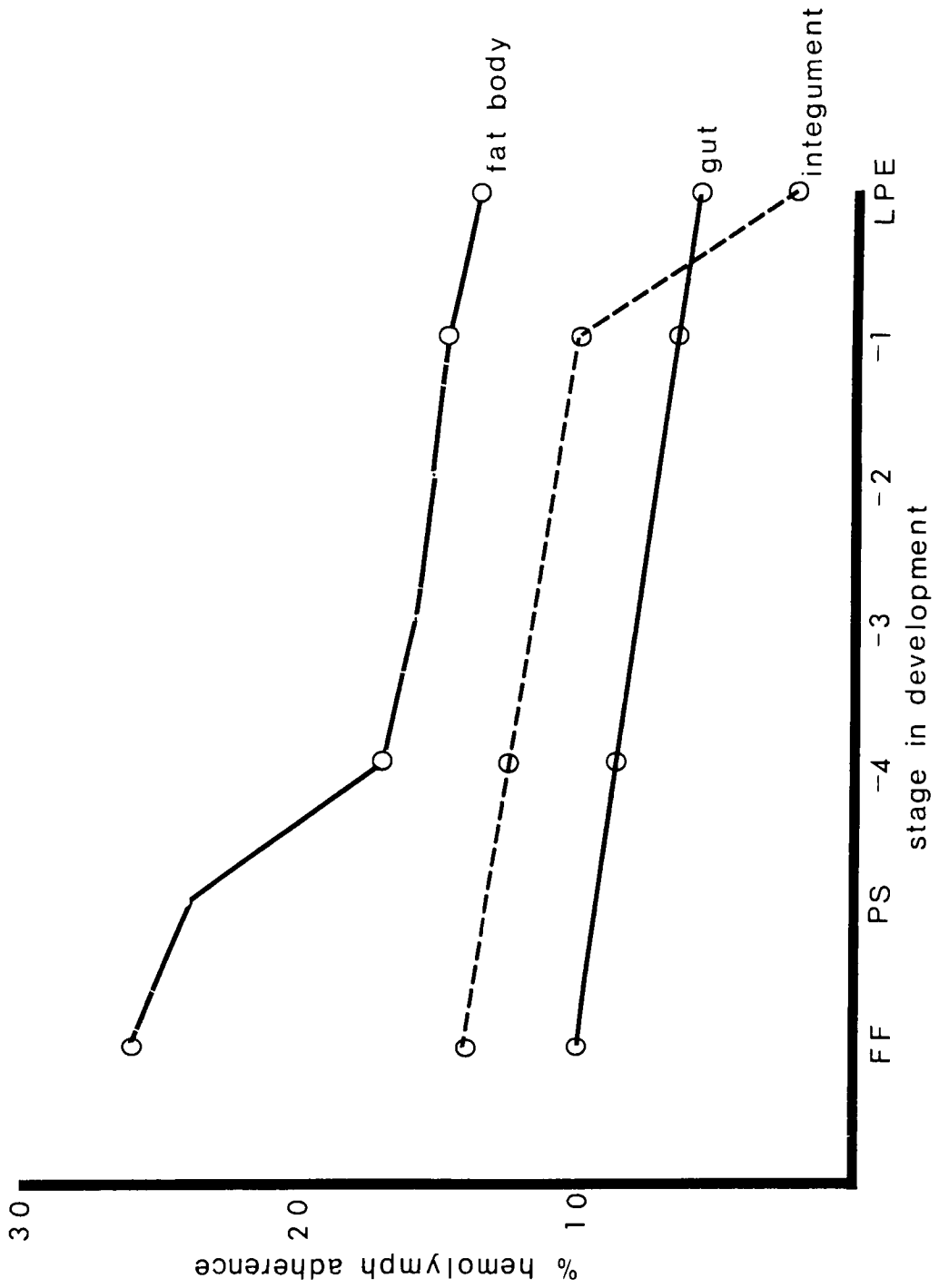
% hemolymph adherence
30
20
10
fat body
gut
integument
FF
PS
-4
-3
-2
-1
LPE
stage in development

Figure 2

Discussion

Total Body Weight. Dramatic losses of body weight during the LPT result primarily from evacuation of the gut contents (FF to -4 days prepupa) and secondly from boring fluid losses (day -4 to -3), while the loss at adult eclosion can be ascribed to the loss of the pupal cuticle and meconium.

The high hydrostatic pressure (late feeding fifth instar) required of LPT animals to facilitate boring into the soil (field reared animals) on days feeding fifth (FF) to pink stripe (PS) is not needed thereafter. Consequently, it is released from the foregut via the mouth and rectum between day PS to -4 day prepupa accompanied by a 30% weight loss (8.5 - 5.9 g). Levenbook, et al. (1971) also noted a 50% decrease in body weight in M. sexta during the LPT, but they attributed this loss to the expulsion of gut contents coupled with a minor loss of water. Their discussion leads one to infer that the water loss is either cuticular or respiratory. While 50% weight losses were also observed in the oak silkmoth, Antheraea pernyi (Fyhn and Saether, 1970) and in the cecropia silkmoth, Hyalophora cecropia (Jungreis and Tojo, 1973), losses in these species can be attributed primarily to the loss of gut contents and secondly to water losses accompanying silk spinning. In restudying weight changes in M. sexta, the volume of secreted boring fluid was measured directly, (data not shown) demonstrating unequivoically that the secondary weight loss is caused by the loss of this fluid and does indeed occur after the animal is placed in its pupation chamber. A description of this phenomena has not been reported heretofore in the literature and will be described

elsewhere (Williams-Boyce, in preparation). Further weight changes occurring after the release of boring fluid may result from increased cuticular permeability and/or from incidential fluid losses occurring during resorption and ecdysis of the larval cuticle. In contrast to the dramatic weight loss during the LPT, the pupal-adult transformation is characterized by a minimal weight loss averaging only 0.3%/day for the 21 day period of development (4.82 - 4.22 g). Weight lost at eclosion (45%) results from ecdysis of the pupal cuticle and release of the meconium, in agreement with observations by earlier investigators working with Manduca sexta (Levenbook, et al. 1971) and the oak silk-moth, Antheraea pernyi (Fyhn and Saether, 1970).

Tissue Weight Changes. The decrease in weight of the integument between the feeding fifth instar larva and the new pupa was only 32% in contrast to losses of 90% observed in H. cecropia (Jungreis and Tojo, 1973). Since M. sexta normally burrows in the earth and is subject to greater abrasions than a pupa enclosed within a cocoon, the need for the thicker pupal cuticle can readily be seen.

While the total body weights, and the weights of the gut contents, gut, integument, and hemolymph all decrease during the LPT, that of fat body increases. In Hyalophora cecropia similar increases (0.9 g to 2.0 g) in fat body weight were recorded during the LPT (Jungreis and Tojo, 1973). In the larvae of starved army worms, Prodenia eridania, Anderson and Patton (1955) observed a 50% decrease in the abdominal fat body content and a 15% decrease in the moisture content of the depleted fat body. In H. cecropia, the water content of fat body cells increases from 50% in larvae to 65% in pupae, (Jungreis and Wyatt, 1972), without an apparent increase in fat cell number (Oberlander, et al., 1965;

Linzen and Wyatt, 1964). The contention is thus supported that a large portion of the weight increase in fat body during the LPT results from increases in intracellular water, storage of the products of tissue histolysis (including urate) and nutritional stores of glycogen, lipids, and protein needed to sustain the animal until eclosion and following adult emergence (Bade and Wyatt, 1962; Locke and Collins, 1965, 1968; Gilbert, 1967 and Wyatt and Pan, 1978). A dramatic loss in hemolymph volume (i.e., most likely lost as the boring fluid) occurs in M. sexta during LPT.

The volume of gut contents decreases following evacuation and excretion of the boring fluid. The sloughed larval midgut epithelium appears as pale orange gut contents at 2 days before the larval-pupal ecdysis. These contents then become dark brown in appearance probably in response to the addition of degradation products from the larval integument and/or oxidation of the contents (Jungreis, 1974). The sequestration of histolytic products by the gut may also be involved in hemolymph osmotic regulation as suggested by Fyhn and Saether (1970) for A. pernyii and Jungreis, et al., (1976) for H. cecropia.

The gut weight decreases approximately 50% from larva to pupa yet the loss at -3 and -2 days before the LPE is 71%, indicating an initial decrease with a subsequent increase. The extracellular space in the midgut epithelium of M. sexta feeding fifth instar larvae occupies 61% of the tissue wet weight (Blankenmeyer, 1977), and if the extracellular space were to continue at this value throughout the LPT, then it would be sufficient to account for increases in gut weight concurrent with increase in gut contents at day -2 to 0 days before

the LPE. In H. cecropia, (Judy and Gilbert, 1970) and M. sexta (Schultz and Jungreis, in preparation) the gut length decreases by some 43% between the feeding fifth larval instar and the new pupa. During this period the foregut and hindgut both collapse to form narrow tubes with degenerated musculature, concomitant with reduction in the size and in the nusculature of the midgut. The loss in gut weight during the LPT is probably due to the breakdown and subsequent loss of its structural components.

The decline in hemolymph volume can be attributed to massive fluid losses associated with expulsion of the boring fluid. The loss of hemolymph volume approaches 61% (2 grams!) during the transformation. In these experiments the M. sexta employed were non-diapausing. Whether such weight losses occur under field conditions or in hornworms entering pupal hibernal diapause is unknown. Several suggestions are pertinent: 1) Manduca prepares a pupation chamber in moist soil which would not require the loss of body water to saturate the air as in those genera which spin a cocoon for a pupation chamber; 2) Manduca loses the boring fluid once it has reached its pupation site which would further serve to humidify the surroundings: 3) the metabolic water gained from possible fat metabolism (Gilmour, 1965; Fyhn and Saether, 1970) during pupation could compensate for a portion of this water loss.

Inulin Study. The decrease in adherence of hemolymph to the integument is probably associated with loss of the extensive larval abdominal musculature. Hemolymph adherence to M. sexta fat body (average 26.5% feeding larvae and 13.5% new pupae) can be contrasted with studies in H. cecropia where hemolymph adherence was an average

of 25% in feeding larvae and 35% in diapause pupae (Jungreis and Wyatt, 1972; Jungreis and Tojo, 1973). Adherence of hemolymph to fat body decreases during the LPT. Visually, one notices an obvious decrease in hemolymph volume following the release of the boring fluid. In fact, losses are so great that the fat body has attained an extremely "viscous" nature by the day of the LPE. New pupae were always analyzed after the larval-pupal ecdysis, a time when a disproportionate part of the hemolymph volume appears to be localized in the head and thorax to facilitate extension of the proboscis and wing sacs. Thus displacement of blood rather than changes in hemolymph osmolality could be responsible for the decline in adhering hemolymph. We were able to determine that the presence of small amounts of gut fluid in gut tissue samples caused some autofluorescence even though we could not correct for this since we did not know the percentage of gut contents in our gut samples. The gut may then have a spuriously high inulin adherence caused by the failure to remove luminal fluids during the drying process. Maddrell and Gardiner (1974) found that the Malpighian tubules of Rhodnius prolixus have a high permeability to inulin. Since the gut tissues used in the these studies were combined with the Malpighian tubules, this permeability to inulin may be another factor responsible for high hemolymph adherence.

CHAPTER II

SYNTHESIS AND STORAGE OF URIC ACID

Introduction

Most terrestrial insects excrete 80-90% of their waste nitrogen as uric acid, although insects of the order Dictyoptera are reported to excrete little uric acid (Mullins and Cochran, 1976). In holometabolous insects, Malphigian tubules or hindgut functions are impaired or lost at the end of each stage in development (Wigglesworth, 1965). As a result, metabolic wastes can not be eliminated from the beginning of the (e.g.), larval-pupal transformation until the day of adult eclosion. This situation necessitates retention of these waste products in non-toxic, osmotically neutral forms, a process termed "storage excretion."

In most insects the major sites if uric acid storage are fat body and integument, storage in integument occurring during active feeding, and storage in fat body occurring preliminary to stage-specific ecdyses (Haydack, 1953; Gupta and Sinha, 1960; Scrivastava and Gupta, 1961; Spiegler, 1962; Kilby, 1963; Tojo, 1971; Jungreis and Tojo, 1973).

Working with extracts of mature tobacco hornworm larvae, Levenbook, et al. (1971) showed that the quantity of uric acid stored in feeding larvae rapidly increases during the larval-pupal transformation. The objectives of this study are (1) to determine which tissue or tissues serve as storage sites for uric acid in the tobacco hornworm, Manduca sexta, and (2) to determine whether this tissue or these

tissues which retain uric acid have the capacity to synthesize uric acid at a rate sufficient to account for that which has accumulated.

Materials and Methods

Determination of Tissue Uric Acid Content. At each stage of the larval-pupal development six to nine animals of approximately equal weight (Figure 1, p. 11) were anesthetized for 15 minutes under 100% CO_2. These animals were divided into 3 subgroups of 2-3 animals and fat body, gut and integument dissected free from the adhering tissues. A few crystals of phenylthiourea were added to inhibit tyrosinase activity. Tissues were blotted on glassine-type weighing paper, the gut contents removed and adhering fat body carefully removed from both gut and integument. These tissues (except for fat body which was used intact) were cut into approximately 1 mm^2 pieces and 6 100-200 mg samples (depending on stage and tissue) weighed to the nearest milligram on a Roller-Smith torsion balance. Tissues were transferred into vials containing 2 ml of chilled (4^oC) 1% lithium carbonate (pH 8.5) and sonicated for 1 minute at 20 second intervals (fat body and gut) or for 2 minutes at 30 second intervals (integument). This treatment caused the rupture of all cells, as confirmed by observation under the light microscope. The uric acid content of integument and gut was determined only at the late feeding fifth and new pupal (day of ecydsis) stages, while the uric acid content of fat body was determined on these and all intermediate days in development.

Uric acid was assayed by a modification of the method of Appelt and Cvancara (1968)(Appendix I). Sonicates of 300 microliters from feeding fifths and 200 microliters from all other stages were

transferred into separate 15-20 ml test tubes containing 0.5 ml of 0.05 M Tris-HCl buffer (pH 8.5) and 2.5 ml of 1% lithium carbonate (pH 8.5). Assays were carried out in duplicate and were of three types: 1) experimental - to which were added 2 milliliters of uricase (0.03 units/ml in 0.05 M Tris-HCl buffer, pH 8.5); 2) control - in which 4 ml of 10% trichloroacetic acid (TCA) were added prior to the addition of uricase, and 3) blanks - uricase was added after incubation. All tubes were then vortexed and incubated without shaking for 8 hours at 37°C in a constant temperature water bath. Reactions were stopped by addition of 4 ml of 10% TCA. Thereafter tubes were centrifuged for 30 minutes at 2000 x g, the supernatants decanted and the absorbances determined at 293 nm. A urate standard curve (see Figure 9, Appendix I) was used to determine the uric acid content of the samples. Tissues urate levels were then determined following correction of the tissue wet weights for adhering hemolymph (Figure 2, p. 15).

Rate of Fat Body Uric Acid Synthesis. The rate of uric acid synthesis by fat body was determined in vitro by measuring the rate of ^{14}C-formate incorporation into ^{14}C-urate (see Appendix I). Duplicate 100 mg quantities of intact fat body from 15 animals (6 feeding fifths and 9 new pupae) were incubated separately for 1 hour at 30°C in 1 milliliter of Jungreis and Wyatt's (1972) modification of Reddy's medium (Stevenson and Wyatt, 1962; Reddy and Wyatt, 1967). The medium contained 5.22 nanomoles of sodium ^{14}C-formate (0.30 µCi) and 0.16 micromoles of unlabeled formic acid. Modified Reddy's medium supplies all of the known precursors for purine synthesis (Heller and Jezewska, 1959) with the exception of CO_2 (produced as an end product of metabolism), APT, and carrier molecules such as formyltetrahydrofolate.

Following addition of ATP to incubates of Antheraea pernyi fat body, Heller and Jezewska (1959) noted a 0.4 - 5.0 fold increase in urate synthesis. Since measurements of the "normal" rather than an "enhanced" rate of uric acid synthesis is intended in these studies, exogenous ATP was excluded from the incubation medium. Reactions were stopped with 100 µl 20% TCA. The incubates were sonicated and five microliter aliquots of the sonicates spotted an activated Gelman ITLC Type SA thin layer plates. The plates were chromatographed in butanol: acetic acid: butyl acetate (2:2:1) until the solvent front had moved a distance of only 3 cm. The plates were then dried in a 109°C oven for 15 minutes to remove the free formate, cut into 1 cm^2 pieces and counted in Econofluor (New England Nuclear).

Results

Determination of Tissue Uric Acid Content. Fat body tissue uric acid (per gram) and total fat body uric acid (per animal) were determined throughout the larval-pupal transformation (Table III). Increases in the absolute amount of stored urate are greatest during the last 3 days of the LPT (excluding the day of ecdysis), with urate content increasing from 18-27 µmoles/day. A 12 fold increase is noted when the data are tabulated as micromoles fat body uric acid/animal which reflects the increase in total fat body wet weight/animal (Figure 3). The urate content per gram fat body tissue doubles between the late feeding fifth larval instar and the pink stripe stage (18 µmoles/g fat body to 48 µmoles/g fat body) and increases 75% between the pink stripe stage and -4 days preecdysis (48 µmoles/g fat body to 82 µmoles/gram fat body). The total quantity of urate present per

TABLE III

CONCENTRATION OF URATE IN THE FAT BODY DURING THE LARVAL-PUPAL TRANSFORMATION

Stage*	# animals	(n)**	µmoles/g f.b.**	µmoles in fat body/animal
1) Feeding fifth	6	3	18.35 ± 0.30	9.58 ± 0.16
2) Pink Stripe	6	3	48.68 ± 3.66	23.79 ± 1.79
3) -4 Days Prior Ecdysis	7	3	82.35 ±12.12	34.92 ± 5.14
4) -3	6	3	100.65 ± 1.77	52.47 ± 0.92
5) -2	9	3	128.56 ± 5.01	85.78 ± 3.34
6) -1	9	3	150.40 ± 8.73	112.78 ± 6.55
7) Day of Ecdysis (Pupa)	9	3	164.59 ±16.18	113.08 ±11.12

*Stages 1-4, 3 pools (2 animals/pool) of fat body with 4 samples/pool
Stages 5-7, 3 pools (3 animals/pool) of fat body with 6 samples/pool
**n = # pools
***Mean ± S.E.

Figure 3. Urate accumulation in fat body of Manduca sexta during the LPT. Values are expressed as micromoles urate stored $\pm$ S.E.$_{\bar{x}}$. Solid line represents micromoles urate/total fat body tissue. Broken line represents microcoles urate/fat body tissue. FF = feeding fifth instar larvae, PS = pink stripe, LPE = larval pupal ecdysis.

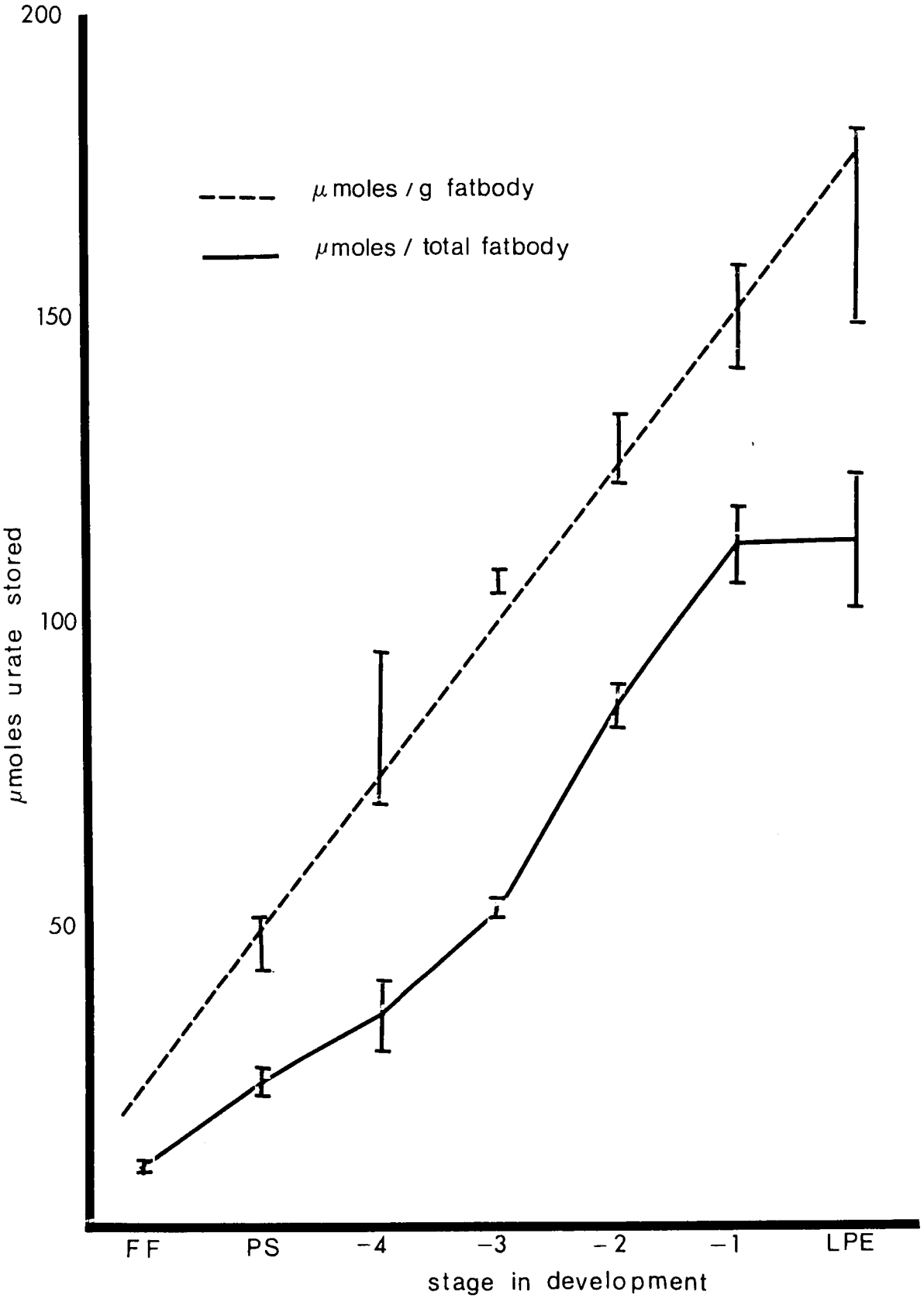
200
150
100
50
μmoles urate stored
μ moles / g fatbody
μmoles / total fatbody
FF
PS
-4
-3
-2
-1
LPE
stage in development

Figure 3

gram fat body again doubles over the next four days (82 μmoles/g fat body at -4 days pre-ecdysis to 164 μmoles/g fat body on the day of the larval-pupal ecdysis). Thus a nine fold increase in fat body uric acid content occurs during this period. The linear increase in stored uric acid calculated per gram tissue is illustrated in Figure 3. It is apparent that a massive increase in uric acid storage occurs in the fat body during the LPT.

Uric acid is distributed primarily in three tissues: fat body, integument and gut, with the largest portion present in fat body (50% in feeding larvae, 83% in new pupae) (Table IV). The total quantity of uric acid in the three tissues averaged 3 mg in feeding fifth instar larva and 23 mg in new pupa (Table IV).

Rate of Fat Body Uric Acid Synthesis. The rates of fat body urate synthesis in vitro in feeding fifth instar larvae and in new pupae were determined and expressed as moles x 10^{-6} of ^{14}C-formate → ^{14}C-urate/g fat body/hr at 30°C (Table V). These rates decreased from a mean of 2.2 μmoles converted/gm/hr to 0.503 μmoles converted/gm/hr, respectively, in feeding fifth instar larvae and new pupae, a minimal 75% drop in synthesizing capacity. For feeding fifth instar larvae, calculated rates probably reflect the minimum urate synthesizing capacity, since substrate saturation of the enzyme was probably not maintained througout the entire period of incubation (data not shown).

Discussion

Levenbook, et al., (1971) have observed that the concentration of uric acid in extracts of whole feeding fifth instar larvae, were 4 to 5 mg per animal, and 57 mg per animal on the day of the larval-

TABLE IV

CHANGES IN MAJOR TISSUE URIC ACID CONCENTRATION

Tissue	Feeding Fifth mg/g	%T**	mg/animal	%T	Pupa mg/g	%T	mg/animal	%T
Total	4.81 ±0.47		2.8 ±0.30		34.24 ± 1.74		22.96 ± 1.39	
Fat Body	3.08 ±0.05	64	1.61 ±0.03	58	27.69 ± 2.72	81	19.01 ± 1.87	83
Gut	1.41 ±0.41	29	0.49 ±0.14	18	4.60 ± 0.91	13	0.81 ± 0.16	4
Integument	0.32 ±0.23	7	0.70 ±0.50	25	1.95 ± 0.94	6	3.14 ± 1.52	14

*1mg = 6 X10^{-6} moles
**%T = percentage of total uric acid found in the three tissues.

TABLE V

URATE SYNTHESIZING CAPACITY IN VITRO

Stage	Micromoles formate incorporated by fat body/hr
Feeding Fifth	2.20 ± 0.45 (n=6)
New Pupa	0.503 ± 0.23 (n=9)

pupal ecdysis. This 11.5 - 14 fold increase correlates well with the 12 fold increase documented in Table II and Figure 3. In addition the total increase in uric acid correlates well with the 12 fold increase in fat body uric acid (1.6 mg → 19 mg, Table IV). Major sites of urate storage apart from fat body could not be located, since urate not present in fat body amounted to only 1.2 mg in feeding larvae and 4.0 mg in new pupae. Values expressed in Table IV are lower than those determined by Levenbook, et al., (1971), a consequence of unmeasured quantities in hemolymph (found to be negligable in H. cecropia, Jungreis and Tojo, 1973, or in Papillio xuthus, Tojo and Hirano, 1968) or in the gut contents, the latter containing urate eliminated via the Malphigian tubules.

Holometabolous insects have been observed to store large quantities of uric acid. It is generally accepted that most of this uric acid is localized in fat body (Leifert, 1935; Anderson and Patton, 1955; Kilby, 1963; Tojo and Hirano, 1968; Jungreis and Tojo, 1973; Lafont and Pennetier, 1975). Levenbook, et al., (1971) proposed the origin of the uric acid found in whole animals to be a product of cuticular proteolysis which must occur preliminary to ecdysis. Earlier researchers working with B. mori hypothesized that uric acid sequestered by fat body is produced at the site of protein degradation, the integument, and only later released into hemolymph where it is readily taken up and stored in fat body (Tsujita and Sakurai, 1965a,b; and Tojo, 1971). In the silkworm, Hyalophora cecropia, Jungreis and Tojo (1973) proved that this idea of uric acid transferral from integument to fat body is incorrect, although they also showed that larval but not pupal fat body readily took up uric acid from hemolymph. Further, Jungreis and

Tojo (1973) showed that in H. cecropia, little uric acid is present in fat body or integument at the time of spinning, but that large quantities were present in the fat body of newly ecdysed pupae. Uric acid was found to be absent from both hemolymph and midgut tissue in H. cecropia, and Jungreis and Tojo (1973) proposed that the uric acid appearing in fat body during the LPT is synthesized de novo in fat body. Studying nitrogen incorporation into uric acid from glycine, aspartic acid and the amide nitrogen of glutamine, Kilby (1963) proposed that the fat body is the site of uric acid synthesis. In the rice stem borer, Chilo suppressalis, Tojo and Hirano (1968) noted that during the period of post-diapause development uric acid accumulated only in fat body (11 mg/g) and in integument (1.9 mg/g), little being present in hemolymph. These data support the contention that the uric acid present in fat body of M. sexta is synthesized de novo. Fat body is also implicated as the major storage site for uric acid in M. sexta, since 58% and 83%, respectively of the total urate found in feeding fifth instar larvae and in newly ecdysed pupae is present in fat body.

The initial quantity of uric acid present in integument of M. sexta feeding larvae is insufficient to account for that which has accumulated in fat body at the time of ecdysis. While Jungreis and Tojo (1973) observed that the quantity of uric acid in integument of H. cecropia did not increase during the larval pupal transformation, an increase was observed in M. sexta, indicating that integument is capable of synthesizing and storing uric acid. In integument precursors for the synthesized uric acid are probably derived from tissues

degenerating during this period. Thus in the tobacco hornworm, fat body is the major site of uric acid storage and is implicated as the major synthesis site.

The evidence that the precursors for the accumulated uric acid originate mainly from amino acids and not purines is supported by several lines of evidence, namely 1) incorporation of labeled amino-acids into fat body uric acid by Periplaneta americana (McEnroe and Forgash, 1957, 1958) Rhodnius prolixus (Barrett and Friend, 1970) and Chilo suppressalis (Tojo and Hirana, 1968); 2) by nutritional experiments a) in which uric acid production was enhanced in the cockroach P. americana (Haydak, 1953) and in the tsetse fly, Glossina morsitans (Bursell, 1965) by rearing insects on diets of high protein content, and b) in which uric acid production was not enhanced by feeding P. americana a diet high in xanthine but was enhanced by starvation (Anderson and Patton, 1955); and 3) by the correlation between nitrogen loss from amino-acids and nitrogen gain in uric acid during metamorphosis of the blowfly, Lucilia cuprina (Birt and Christian, 1969), in the silkworm, Bombyx mori (Tojo, 1971) and in the rice stem borer Chila suppressalis (Tojo and Hirana, 1968). According to Bursell (1967) it has long been accepted as fact that the large amounts of uric acid which occur in the excreta of insects cannot derive solely from nucleic acid degradation. The levels of nucleic acids in most insects are too low to account for more than a small fraction of the stored uric acid. Anderson and Patton (1955) did show the presence of adenase and guanase in larvae of the army worm, Prodenia eridania and the meal worm Tenebris molitor, from which I infer that fat body can produce uric acid from performed purines. However, based on data

for these two species the amount produced compresses only 2 to 20% of the total stored urate.

Avise and McDonald (1974) examined the activities of numerous enzymes in whole body preparations of Drosophila pseudoabscura and found decreasing enzyme activities from larva to pupa with most enzymes recovering to larva levels or higher in adults. A decrease in the activity of xanthine dehydrogenase was noted at the time of the larval-pupal ecdysis in Pieris brassicae (Lafont and Pennetier, 1975) and in the Japanese beetle, Popillia japonica (Ross, 1959). A low level of RNA polymerase activity was found in fat body of M. sexta and in H. cecropia at the time of the larval-pupal ecdysis (Sridhara and Gilbert, 1975; Berry, et al., 1967). These authors suggest that the decreasing enzyme levels in pupae may reflect gene repression, increased degradation (or inactivation) of existing enzyme molecules or both. The higher rate of fat body uric acid synthesis observed in feeding fifth instar larvae can be contrasted with that of new pupae (Table V, p. 30) but may be related only to the activity of xanthine oxidase ($\text{Xanthine}_{XO} \rightarrow$ hypoxanthine $\rightarrow_{XO}$ uric acid) at the respective stages. Xanthine oxidase could be inhibited by increasing amounts of uric acid in fat body even though a large portion of the stored uric acid may be in crystalline form (Fried and Fried, 1974; Tojo, cited in Wyatt, 1975). Further, Bergel (1961) has showed that xanthine oxidase is a rate limiting step in purine metabolism. It follows that the rate of uric acid synthesis measured at the beginning of the LPT would appear to be higher, since little accumulated uric acid is present in fat body. Yet, in consideration of the evidence for a general decrease in enzymatic

activity to pupation the lower rate of urate synthesis found in the new pupa may reflect a decrease in enzymes apart from xanthine oxidase.

The in vitro rate of urate synthesis on any given day of the LPT (using either the synthesizing capacity of the system or the total amount of urate accumulated, whichever was less) can account for 45% of the accumulated urate. It must also be considered that the rate study was performed at 3-5°C higher than the rearing temperature which would increase the rate of synthesis. Therefore in view of this increase in temperature, the in vitro system can account for approximately 35% of the urate accumulated during the LPT. Regardless, the decrease in the in vitro synthesis rate with the advancing LPT is believed to be a real reflection of the normal pattern of synthesis though by no means equalling the normal rate. It is also felt that this decrease in the formate incorporation is not due to an increase in the formate endogenous pool since the endogenous levels of co-factors have been shown to remain relatively constant, and since formate is not the first precursor of the purine ring. The precursor pools could have been measured but this would only have confirmed the total amount of precursors available for all the cellular reactions, not just for uric acid production. Further investigations, outside the range of this study, would be necessary to elucidate this point.

In a major review of insect excretory systems and mechanisms, (Maddrell, 1971), paid scant attention to storage excretion. Maddrell uses this term only in reference to those compounds which will be used by the animal at a later stage in development. Uric acid is assigned a function as a useless excretory product and is viewed as deposit versus

storage secretion (i.e., the situation encountered in hemi-metabolic insects). However, various functions for the stored uric acid have been proposed: 1) as a store of nitrogen for use in the production of new tissues or for when the insect is in negative nitrogen balance (Ludwig, 1954; Tojo and Yushima, 1972); and 2) to provide a source of hypoxanthine for the production of nucleotides (Ross, 1959; Kilby 1963).

In the Japanese beetle Popillia japonica uricase could not be detected after the early prepupal stage. However, the quantity of stored uric acid decreased during late pharate-adult development concomitant with a rise in xanthine oxidase activity (Ross, 1959). In the boll weevil, Anthonomus grandis, Mitlin and Maulkin (1966) observed that isotopically labeled uric acid was incorporated into six free amino acids, suggesting a use for uric acid as a source of dietary nitrogen in newly emerged adults. When reared on a low-protein diet, Haydack (1953) found that the fat body uric acid content of adult P. americana dropped markedly, without subsequent excretion. When labeled uric acid was administered to adult P. americana , McEnroe (1959) found that during the first 24 hours post injection, 8% of the injected labeled could be recovered as respiratory $^{14}CO_2$. Unfortunately, the contribution of microbial degradation of uric acid to the total oxidized was not determined. In fat body of Periplaneta orientalis, Keller (1950) and Toth (1952) have both independently shown the presence of symbiotic bacteria which metabolize uric acid to provide nitrogenous compounds for host anabolism. According to Gilmour (1965) no insect retains the full complement of enzymes needed

to degrade uric acid nitrogen to the level of ammonia. Levenbook, et al., (1971) found that the total uric acid in Manduca sexta decreased by about 50% prior to the pupal-adult apolysis. According to Gilmour, these products of purine catabolism enter the metabolic cycles are ribotides, which may then be metabolized by reversal of purine synthesis (McEnroe and Forgash, 1958).

Stored uric acid also functions as precursors of nitrogen containing pigments such as pterines. According to Chefurka (1965b) the fall in concentration of uric acid in the pupa of Drosphilia may be connected to the time when the eye pigments are synthesized. Tojo and Yushima (1972) found that one half of all the uric acid present in pupae of the swallowtail butterfly, Papilio xuthus, and the cabbage butterfly Pieris rapae crucivora was retained in the adult. They determined that uric acid was present in the white portion of the wings and that a uric acid metabolite could be found in the black parts of adult wings. In the cotton stainer Dysdercus fasciatus, Berridge (1965b) found that permanent stores of uric acid accumulate in the epidermis and contribute to the color pattern of the insect. The progressive increase in uric acid throughout the insects life accounts for increases in white markings in later instars of this insect. In Pieris brassicae, LaFont and Pennetier (1975) showed that fat body uric and allantoic acids were transported into the wings and gut of developing pharate adults.

Uric acid may simply be deposited as a nitrogenous waste product during the larval-pupal transformation when excretion is restricted and quantiatively excreted at adult eclosion, as is observed in the silkworm Bombyx mori (Tojo, 1971). In those insects in which the

Malpighian tubules are not the primary organ of excretion, uric acid is found to accumulate in fat body throughout development. In these species, uric acid is eliminated through urate cells of the anterior intestine, with the remainder stored in fat body (e.g. the cockroach P. americana, Srivastava and Gupta, 1961). Storage of uric acid in the accessory glands of male cockroachs is another example of this type of storage excretion. In the German cockroach, Blattella germanica, uric acid is stored in the utriculi majores of the accessory glands of the male reproductive system. When two animals copulate the uric acid is released by the male and covers the spermatophore and parts of both the male and female. Its exact function in copulation is unknown but it is suggested that the accessory glands may have a dual role in reproduction and excretion (Roth and Dateo, 1964, 1965; and Roth, 1967).

CHAPTER III

CONTROL OF URIC ACID SYNTHESIS

Introduction

The endocrine physiology of M. sexta during the larval-pupal transformation has been described in considerable detail regarding ecdysiotropin (=Prothoracicotropin = PTTH) and Juvenile Hormone (JH) (Nijhout and Williams, 1974 a,b); JH and ecdysone control of epidermal cell commitment, Riddiford (1976 a,b); Ecdysone metabolism, King (1972); Ecdysone and RNA polymerase activity, Sridhara and Gilbert (1975); ecdysone and JH, Nijhout (1976); temporal control of PTTH release, Truman and Riddiford (1974); ecdysone and pupal cuticle sensitivity, Mitsui and Riddiford (1976); ecdysone titers and prothorocic gland activity, Bollenbacher, et al., (1975). However the mechanism by which ecdysone elicits the diverse array of physiological and biochemical responses culminating in ecdysis is essentially unknown. Seemingly, ecdysone, in controlling the series of complex morphogenetic changes occurring to ecdysis, might also control the subsequent set of metabolic changes needed to handle accumulating waste nitrogen now that the insect has entered a closed system for the completion of metamorphosis.

The findings of Bodenstein (1953) and Thomas and Nation (1966) using Periplaneta americana, and Mordue and Goldsworthy (1973), using Locusta migratoria and Schistocerca gregaria, suggest that the corpora cardiaca and the neurosecretory cells of the brain are more important in uric acid metabolism than the corpora allata. These observations

implicate a causal relationship between hormonal activity and metabolism. In order to elucidate these relationships, experiments were designed to determine whether storage or synthesis of urate by fat body is under hormonal control.

Materials and Methods

Animals. Experiments were conducted with larvae of the tobacco hornworm, Manduca sexta, from our laboratory colony during the larval-pupal transformation (see Chapter I).

In M. sexta the rate of fat body urate synthesis is the same regardless of the origin within the body of the fat body tissue (data not shown). By ligating the animals the posterior section is isolated from a changing hormonal environment since the anterior section contains the major endocrine organs involved in metamorphosis.

Ligation. To determine if there was a hormonal influence on uric acid synthesis and accumulation in the fat body tissue, thirty-five animals in the pink stripe stage were CO_2 anesthetized and ligated between the fifth and sixth abdominal segments, because this region permitted equal division of the animals fat body tissue. After ligation they were placed in covered plastic containers, since the survival rate of ligated animals was found to increase considerably by allowing them freedom of movement to facilitate circulation. On the day of the ligation (+ 4 hours) and for each day of the transformation thereafter, 5-6 animals were removed from the group for analysis. These animals were CO_2 anesthetized, bled, and the fat body dissected for each section. Duplicate 100 mg samples from each section of the ligate were placed in 1 ml Reddy's medium with 5.22 nanomoles sodium

^{14}C-formate and 0.16 micromoles of cold formate. The samples were incubated at 30°C in a water bath for 1.5 to 3.0 hours with frequent, gentle agitation to allow the fat body to remain suspended in the medium. At the end of the incubation period the reaction was stopped with 100 µl of 20% TCA, and the samples sonicated for 1 minute at 20 second intervals. Activated siliac acid plates were spotted with 5 µl of the sonicates and chromatographed for 3 cm with butanol: acetic acid; butyl-acetate (2:2:1). The plates were dried in a 109° oven for 15 minutes to remove the free formate, cut into 1 cm pieces, placed in 3 ml of toluene phosphour, and counted for 5 minutes per sample. The rate of conversion of formate was then calculated per gram fat body tissue/hr and the anterior and posterior values compared.

Ligation with Ecdysone Administration. Twenty-five pink stripe animals were CO_2 anesthetized, of these, 15 were ligated, weighed and injected in the last abdominal segment with ecdysone (10µg/ml hemolymph) in 0.1 M KCl (saturated with phenylthiourea and steptomycin) and the remaining 10 injected with the KCl carrier. At 2 and 4 days after treatment, the animals were reanesthetized, dissected and the rate of uric acid synthesis determined as in the previous ligation study. The data was calculated as paired ratios of the posterior/anterior samples, and subjected to the student's statistical "t" test, with a confidence limit of $p<0.05$.

Ecdysone Administration to Non-Ligates. One hundred and twenty-five feeding fifth instar animals were divided into groups of 10-20 with each group receiving daily injections of ecdysone, 10µg/ml hemolymph, or KCl carrier) initiated at successive stages of the transformation. All animals were analyzed on the expected day of pupation.

From each group of ten animals fat body tissue from 3-5 was pooled and five 200 mg samples were taken from each pool. Of the 5 samples, one was TCA precipitated at 0 minutes incubation, 2 were incubated for 1 hour and 2 were incubated for 2 hours. The assay was performed as in the ligation study except that 50 µl samples of the sonicates were placed on 2 cm^2 pieces of siliac acid plates, 50 µl of 20% TCA were added directly to the sample spot, and the plates were heated for 15 minutes at 109°C to volatilize the remaining labeled formate. The samples were then counted and the data calculated as formate converted/hr/gram fat body tissue.

Accumulation of Uric Acid in Ecdysone Administered Non-Ligates. Triplicate 100-300 ul samples of the sonicates from the previous study were placed in 6-9 ml of 1% Li_2CO_3, pH 8.5, protein precipitated with 20% TCA, the samples centrifuged for 1 minute on a Beckman microfuge (Model B), and the supernatant was pipeted free of the lipid cap layer and protein pellet. An additional two samples of the sonicates from each pool were analyzed via the modified uricase method to assure the accuracy of the simple procedure (see Appendix I). All of the supernatants were then read spectrophotometrically and the amount of urate determined from the standard curve.

Results

Ligation. The in vitro urate synthesizing capacity of the fat body was determined for both the anterior and posterior sections of ligated animals. Figure 4 shows the "normal" or anterior rate of uric acid synthesis during the transformation. The values at the pink stripe stage and new pupal stage correlate well with our findings

Figure 4. Normal pattern of uric acid synthesis in Manduca sexta during the LPT. Values are from the anterior portion of ligated animals, each value the mean of 5-6 animals ± S.E. PS = pink stripe, LPE = larval-pupal ecdysis.

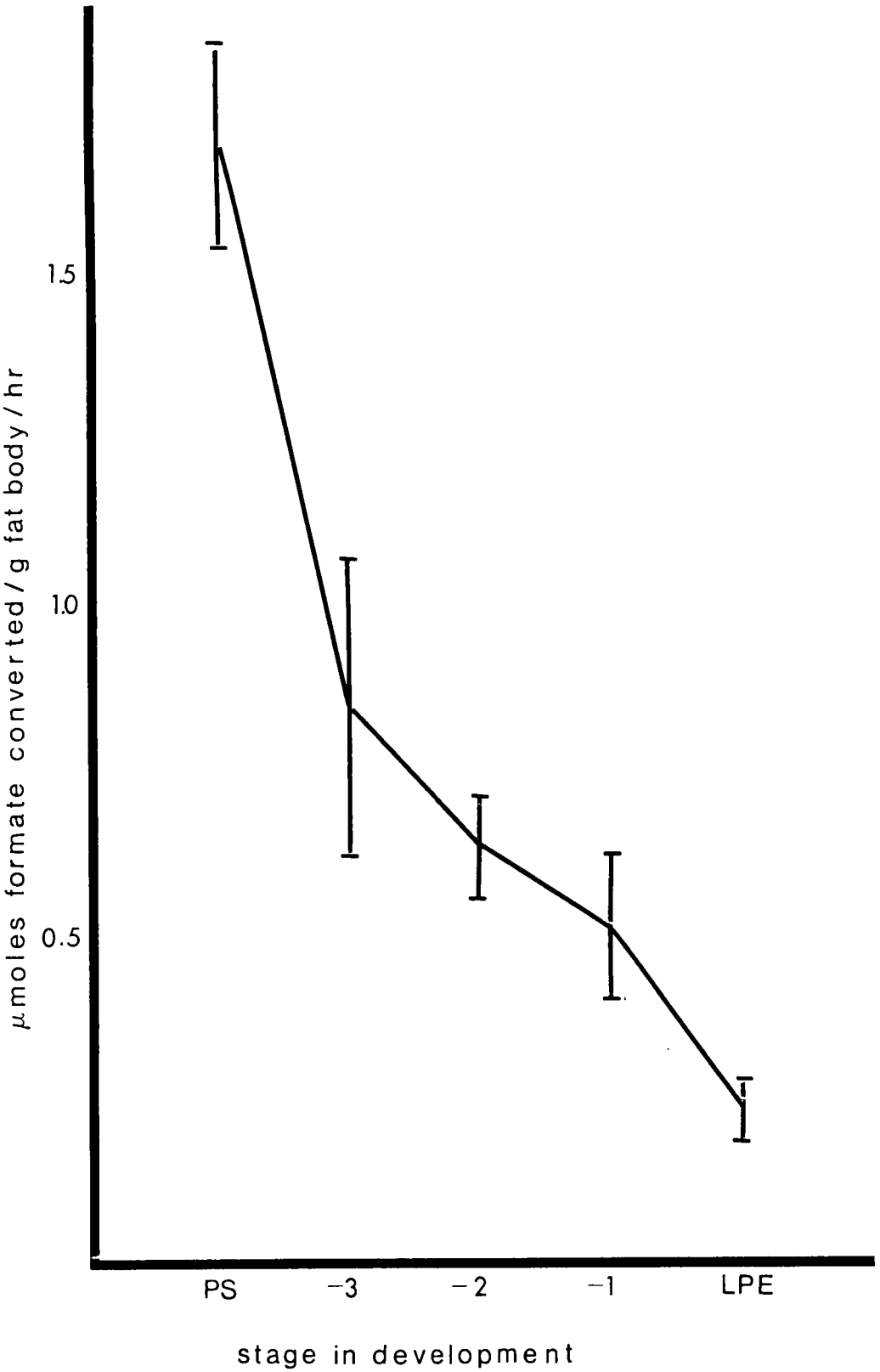
1.5
1.0
0.5
μmoles formate converted / g fat body / hr
PS
−3
−2
−1
LPE
stage in development

Figure 4

from unligated animals (unligated feeding fifth = 2.20 ±0.45 micromoles formate/hr/gram tissue, ligated pink stripe = 1.69 ±0.16; unligated pupa =0.503 ± 0.23, ligated pupal stage .231 ± 0.05). The most dramatic decrease in rate of synthesis (50%) occurs between the pink stripe stage and -3 days prepupa.

Table VI lists the synthesis rates for both the anterior and posterior sections of ligated Manduca sexta. Very little difference is seen between the anterior and posterior rates of formate incorporation at the pink stripe (1.69 - 1.62) and -3 days prepupal stages (.843 - .730). While the anterior rates follow the normal decline (37% of pink stripe rate at day -2 prepupae) the posterior value is 67% (at day -2 prepupa) of the pink stripe rate. It maintains this rate until the expected day of pupation, at which time the rate falls to 42% of the pink stripe rate, whereas the anterior rate is only 14% of the pink stripe rate.

Paired data of the rates, posterior/anterior, show dramatically the difference between the two sections of the animal (Figure 5). The sections are at unity for the first two days (.9856-.9588) then a linear increase in the ratio is seen (.9588-3.25) for the remainder of the LPT. Morphologically, the anterior portion of the animal showed some underlying pupal cuticle tanning but the animal could not experience ecdysis. The posterior section continued to appear as a -3 day preecdysis animal.

Ligation with Ecdysone Administration. When posterior/anterior paired ratios were compared between ecdysone injected experimentals and KCl injected controls it was found that the difference was highly

TABLE VI

URATE SYNTHESIZING CAPACITY IN LIGATED ANIMALS

Stage	Formate incorporated/hr/g fat body	
	Anterior	Posterior
Pink Stripe	1.69 * ± 0.16 (n=5)	1.62 ± 0.12 (n=5)
-3 Days Prepupa	0.84 ± 0.22 (n=5)	0.73 ± 0.15 (n=5)
-2	0.63 ± 0.08 (n=6)	1.09 ± 0.24 (n=6)
-1	0.51 ± 0.11 (n=6)	1.01 ± 0.12 (n=6)
Pupa	0.23 ± 0.05 (n=5)	0.69 ± 0.16 (n=5)

*umoles ± S.E. of the mean

Figure 5. Paired data of urate synthesis in ligated Manduca sexta during the LPT. Values are ratios of posterior rate/anterior rate $\pm$ S.E.$_{\bar{x}}$. Numbers = paired observations. PS = pink stripe larva, LPE = larval-pupal ecdysis.

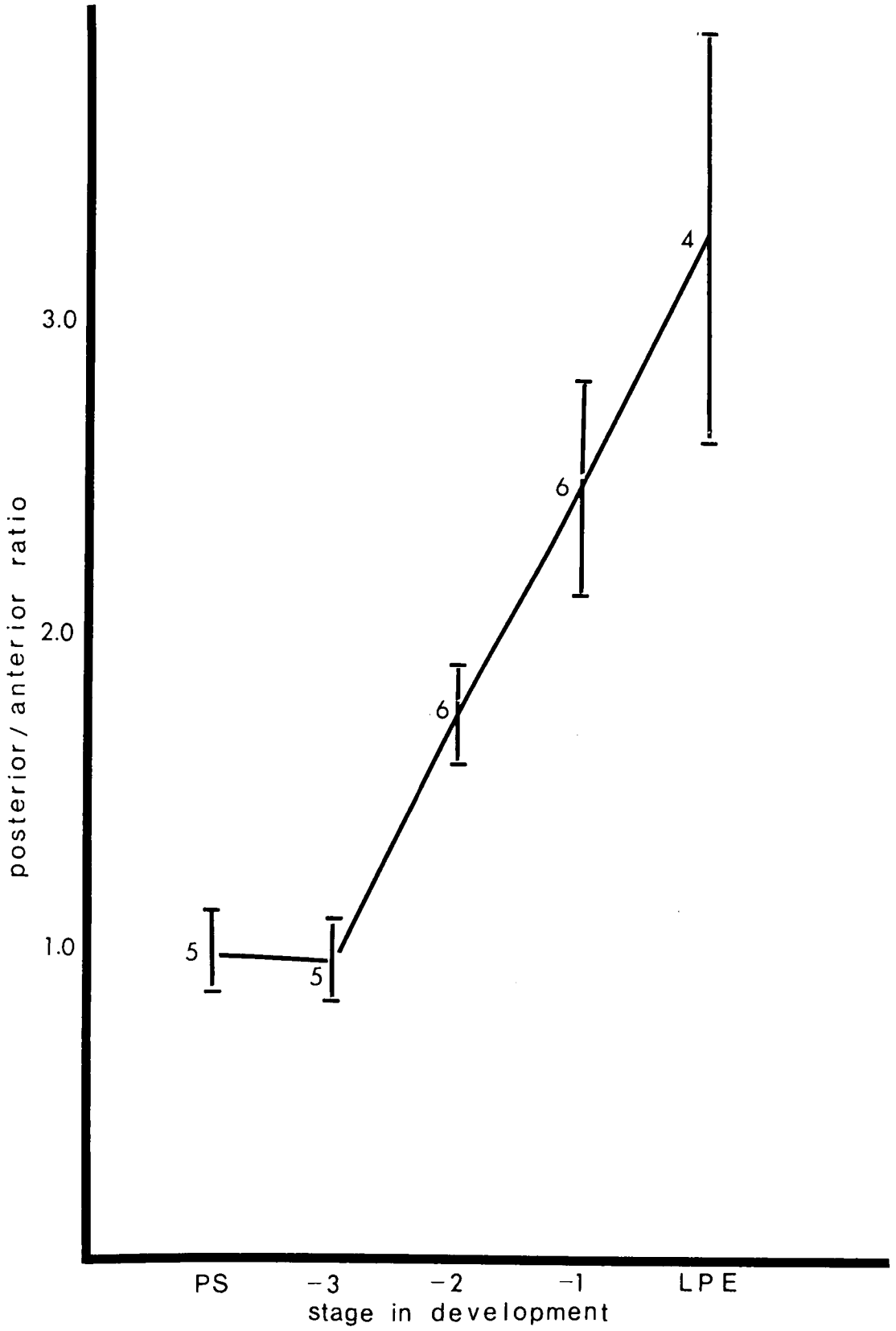
posterior / anterior ratio
3.0
2.0
1.0
5
5
6
6
4
PS
−3
−2
−1
LPE
stage in development

Figure 5

significant ($p<0.001$) for both 2 and 4 days post ecdysone injection (Table VII). Paired ratios from ecdysone administered animals are identical to pink stripe non-hormone administered ligated animals ($p<0.05$). Morphologically there was very little difference between the anterior and posterior sections of the animal, both showed underlying pupal cuticle tanning but neither experienced ecdysis.

Ecdysone Administration to Non-ligates. The fat body of pupae whose injections started on one of the first 4 days of the transformation retained its larval capacity to synthesize urate, an average 1.5 μmoles formate incorporated/hr/g tissue. Animals whose ecdysone injections were initiated on days -2 or day of pupation and all injected controls expressed the normal non-ligated pupal rate of synthesis, an average 0.57 μmoles formate incorporated/hr/g tissue (Figure 6).

Accumulation of Uric Acid in Ecdysone Administered to Non-Ligates. No significant difference was found between the amount of stored urate in the fat body of both ecdysone ($\bar{x} = 206 \pm 12$) and KCl - injected pupae ($\bar{x} = 186 \pm 16$), (Figure 7) and the amount stored in the fat body of non-ligated pupae ($\bar{x} = 165 \pm 16$), (Figure 3, p. 27). However, a slight elevation ($p<0.05$) in stored urate was seen between non-ligated pupae and controls and experimentals injected on the first 3 days of the transformation, 200-250 μmoles/g. Only one point (day -2) deviated between controls and experimentals.

Discussion

Previously, little research has been carried out in relation to waste nitrogen output and hormonal effects upon nitrogen metabolism. By using ligation experiments it was the aim to gather certain

TABLE VII

EFFECT OF ECDYSONE ON URATE SYNTHESIS IN LIGATED ANIMALS

Days After Ecdysone Administration	Posterior/Anterior Paired Ratios		P
	Control KCl Injected	Experimental Ecdysone Injected	
2 Days (-2 Days Prepupa)	1.79 ± 0.14 (n=6)	1.28 ± 0.21 (n=8)	($p<0.001$)
4 Days (Pupa)	3.25 ± 0.66 (n=4)	1.196 ± 0.057 (n=4)	($p<0.001$)
Uninjected Pink Stripe	0.986 ± 0.118 (n=5)		

Figure 6. Effect of exogenous ecdysone on urate synthesis in unligated Manduca sexta. Values are μmoles formate converted/hr/g fat body tissue ± S.E.$\bar{x}$. Solid line ecdysone injected, broken line KCl carrier. Control injected. FF = feeding fifth larvae, PS = pink stripe larvae, LPE = larval-pupal ecdysis.

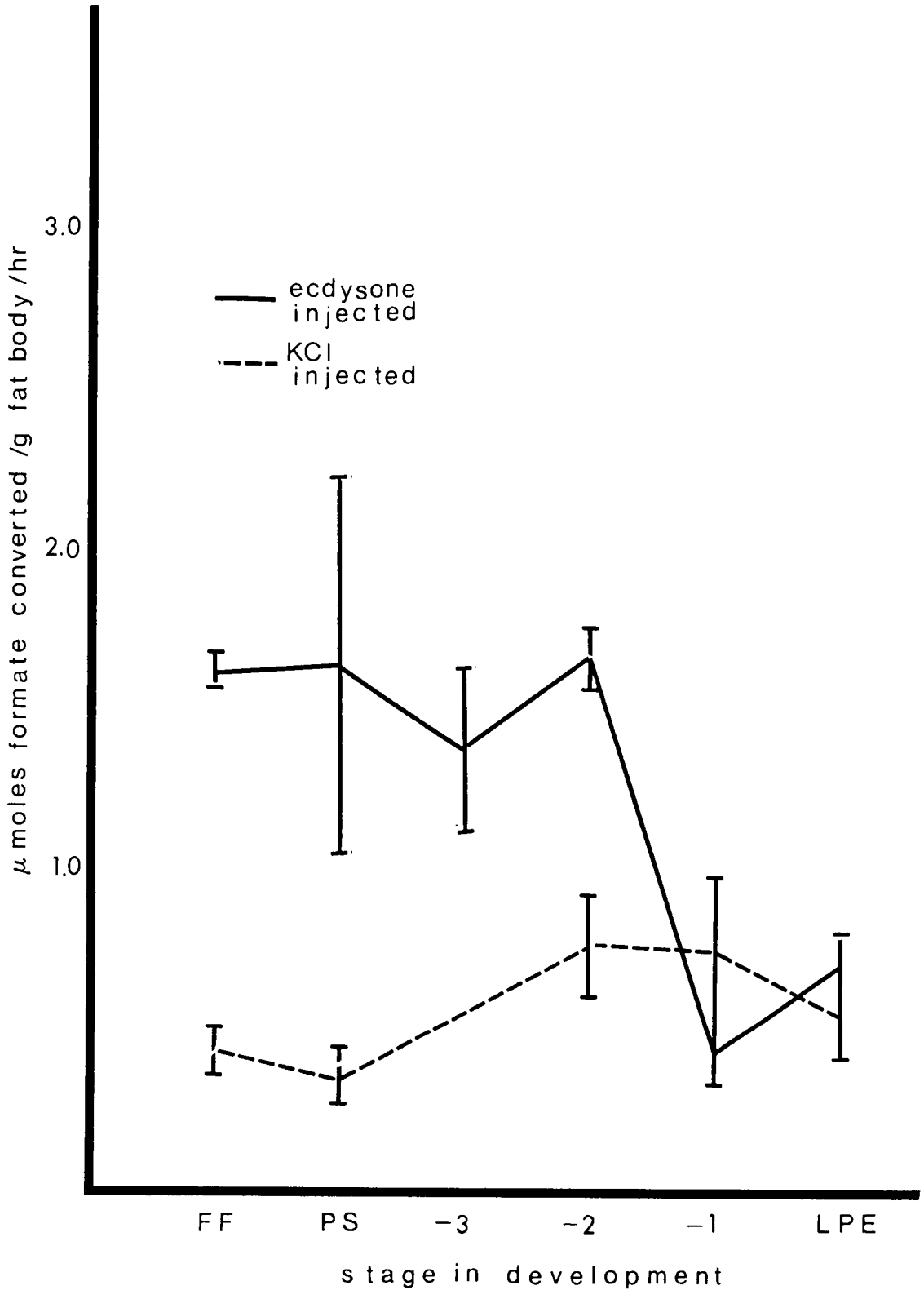
3.0
2.0
1.0
μ moles formate converted /g fat body /hr
ecdysone injected
KCl injected
FF
PS
−3
−2
−1
LPE
stage in development

Figure 6

Figure 7. Uric acid accumulation is unligated Manduca sexta under ecdysone treatment. Values are μmoles urate accumulated per gram fat body tissue $\pm$ S.E.$_{\bar{x}}$. Solid line ecdysone injected, broken line KCl carrier injected control. FF = feeding fifth larvae, PS = pink stripe larvae, LPE = larval-pupal ecdysis.

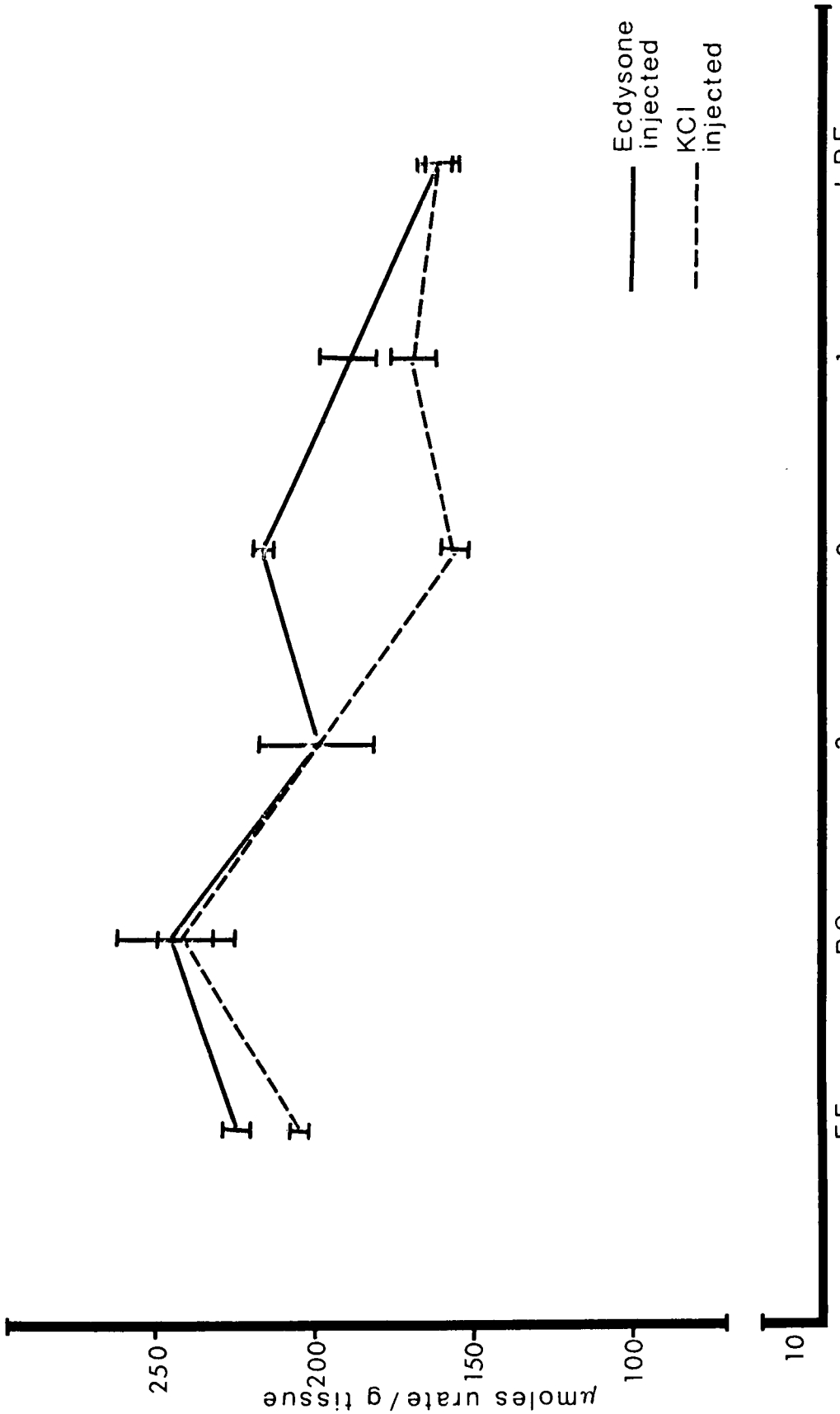
Ecdysone injected
KCl injected
μmoles urate / g tissue
250
200
150
100
10
FF
PS
-3
-2
-1
LPE
stage in development

Figure 7

circumstantial evidence for the involvement of hormones in uric acid metabolism. In the anterior section of ligated animals the rate or uric acid synthesis followed the same pattern as the nonligated animals which confirmed the established use of the anterior tissues of ligates as controls. The comparison of the anterior and posterior rates was striking in that the posterior fat body showed a rate of urate synthesis which was 40-70% greater than that of the anterior fat body of the same day, and 65% of the pink stripe rate. The initial drop at day -3 in the posterior section could be attributed to the first peak of ecdysone, (Bollenbacher, et al., 1975, see discussion below). From these results it is apparent that the absence of some endocrine influence under ligation allowed the rate of urate synthesis to be maintained at a higher level, suggesting hormonal metabolic control.

A small number of other investigators have delved into hormonal influence on nitrogen metabolism. Bodenstein (1953) in the course of studying allatectomized adult roaches, Periplaneta americana, noticed that their fat body differed from the fat body of normal individuals. Investigation of this phenomena showed that removal of the corpora allata and corpora cardiaca from adult animals resulted in the disappearance of urates from the fat body which could be restored by retransplantation of the corpora cardiaca. Bodenstein suggested that the corpora cardiaca regulates either the breakdown of protein which would alter urate metabolism and/or the mechanism that mobilizes urates. Thomas and Nation (1966) found that allatectomy during an ovarian cycle in P. americana females results in a lower level of total body RNA and protein, and that total body uric acid failed to increase as

it does in control females, supporting Bodensteins observations that allatectomy has no influence on stored urates. The relationship between allatectomy and uric acid was also studied by Mordue and Goldsworthy (1973) in the adult locusts, Locusta migratoria and Schistocerca gregaria. Allatectomy once again proved to have little effect on uric acid synthesis, except for a 4-5 day elevation. They did find that removal of the neurosecretory cells resulted in a very low level of uric acid production with a drastic reduction in the levels of two transaminase (key enzymes in the formation of non-essential amino acids, in metabolism of waste nitrogen, and in gluconeogenesis) enzymes, GPT and GOT, in fat body. These data suggest that the control of enzymes involved in uric acid metabolism and the subsequent production of uric acid are under the control of neurosecretory cells (Wadano and Miura, 1976; Sridhara and Gilbert, 1975; Avise and McDonald, 1976).

The evidence supporting the corpora cardiaca's involvement in nitrogen metabolism suggests a hormonal control of uric acid production. The site of this control could be in the fat body since it has been shown to be a target organ for ecdysone (Gilbert and King, 1973) and possesses the ability to convert α (prohormone) to β (active hormone) ecdysone (King, 1972). According to Bollenbacker, et al., (1975) there are 2 peaks of ecdysone titer during development of the fifth instar larva to the pupa in Manduca sexta. The first small peak occurs between the day of the late feeding fifth instar and the pink stripe stage, eliciting the changes which characterize the switch over from the larval to the pupal development. The second peak, 8 fold higher than the first ecdysone titer, occurs at day -2 prepupa and marks the beginning of the pharate pupal development (apolysis to

pupation). The prothoracic gland secretory activity peaks at day 4-5 (FF to pink stripe) and on day -3 prepupa. The animals in this study were ligated after the first, smaller peak in ecdysone titer, and it can be inferred that the one injection of ecdysone triggered some pattern of metabolic events similar to the second peak of ecdysone. Bollenbacher, et al., (1975) found that the second peak of ecdysone titer had a concentration of 1.4 µg/ml while our injected ecdysone was 10 µg/ml a seven fold increase above the physiological range for that day. However, Nijhout (1976) working with M. sexta deemed that small doses of β-ecdysone given over a long period of time, were more effective than a single large dose containing more ecdysone than the combined small doses (a single dose being inactivated before it could elicit a full response). Yet some responses only need a few hours of hormonal exposure (Cherbas and Cherbas, 1970).

In this study, the ability of tissues to respond to juvenile hormone was not tested since Nijhout and Williams (1974 a,b) and Riddiford (1976) could not detect JH in M. sexta larvae heavier than 7.5 g. Also Truman and Riddiford (1974) with studies in M. sexta established that ecdysone alone is responsible for the initiation of and the continuation to pupation. Since this was directed at the LPT when the level of JH is undetectable, it was felt that JH might not play a direct role in the control of uric acid production during this period of development. For these reasons, a study of JH effect was not within the scope of this project.

When the effect of daily doses (starting at successive days of the LPT) of ecdysone (each dose seven fold over physiological range)

were studied in unligated pupae the rate of urate synthesis increased and/or was maintained at the larval capacity of synthesis for animals receiving ecdysone injections prior to the -1 day prepupal stage. At stages -1 prepupal and new pupal, exogenous ecdysone was without effect and these values are in complete agreement with the KCl carrier controls. This alteration in synthesis rate with 7-40 times the physiological dose (not including endogenous contribution) is not surprising. It is well illustrated in clinical studies of tumor patients that the same hormones (steriods, as is ecdysone) which enchance tumor growth can also cause the tumor to regress when used in great excess of the physiological dosages. It is also documented that estrogens increase protein synthesis, and if the rate of protein synthesis and estrogen concentration are plotted against the increase in pharmacological dose a bell shaped curve is obtained indicating a reversal of the hormonal effect. The current theory of explanation for this phenomena is that the cytoplasmic receptor which has entered the nucleus is not released (i.e., the normal effect is first seen, but continuation of this effect is inhibited). Since the receptor has to be motile to function, any further response to the hormone is thus prevented until new receptor is produced. In our studies the fat body from all animals was analyzed on the day of pupation, after having experienced varying dosages of ecdysone depending on the day the injections began. It is reasonable to assume that the increased rate or uric acid synthesis seen in the four groups of animals which had been injected for the longest period of time was due to a blockage of ecdysone action. Animals injected for only 1-2 days could respond to the total lower amounts of hormone and exhibited the normal rate of synthesis.

The mechanisms through which ecdysone effects the rate of urate synthesis are not known. It is considered that ecdysone acts as a gene-activating agent. Its primary influence on the organism is the stimulation of synthesis of all RNA types (Beerman, 1959; Stevenson and Wyatt, 1962; Gilbert, 1964; Barth, et al., 1964; Clever, 1965; Berry, et al., 1967; Wigglesworth, 1970; Cherbas and Cherbas, 1970; Bar-Zee and Kaulenas, 1975), and DNA synthesis (Krishnakumaran, et al., 1967). Ectodermal tissues are found to be the main target for ecdysone, though an additional target is the fat body tissue (King, 1972). This rate enhancement of RNA and DNA synthesis may produce alterations in concentrations of enzymes of purine synthesis, increase enzyme inactivators, or simply by enhancing nucleic acid synthesis (with the subsequent increase in protein synthesis) remove a substantial portion of free amino and/or nucleic acids from the cytoplasmic pool resulting in a decrease in precursors for uric acid synthesis.

The samples which showed high rates or uric acid synthesis under the influence of ecdysone were subjected to uric acid analysis and the amount of stored uric acid was found to be equal to that amount stored in the KCl injected controls or normal pupae. Since activities of individual enzymes, or of sequences of enzymatic reactions are commonly regulated by the (1) availability of substrate (2) coenzymes, (3) concentration of reaction products, it is reasonable to propose from this study that fat body urate production is regulated during the LPT by precursor availability and not by changes in urate synthesing capacity which is under ecdysone control.

SUMMARY

The larval-pupal transformation (LPT) in the tobacco hornworm, Manduca sexta, as in other lepidopterous insects, heralds the beginning of numerous metamorphic changes which will culminate in the formation of an adult. During this transformation M. sexta decreases in weight from 9.16 g to 4.87 g. This total weight loss is reflected by the decrease in weight of all tissues of the animal with the exception of the fat body tissue. The absolute quantity of fat body increases from 0.5 g to 0.75 g during the LPT. The quantity of uric acid retained by the animal increases from 17 → 137 micromoles during this same period, with the majority of the urate associated with the fat body (9 → 113 μmoles). When the capacity of intact cells to incorporate ^{14}C-formate into uric acid was measured in vitro (assay established by this study), a decline in rate from 2.2 to 0.05 micromoles/g fat body/hr at 30°C was noted, even though storage deposition or total urate concentration was accelerating during this period. The ligation of pink stripe animals revealed hormonal control of urate synthesizing capacity, with the posterior sections expressing (on the day of pupation) a 3 fold increase in retained urate synthesizing capacity relative to the anterior sections. The addition of ecdysone to the posterior sections of ligates showed a restoration of the normal relationship between anterior and posterior sections. Ecdysone administered to non-ligated animals on successive days of the LPT and measured on the day of ecdysis, prevented a loss in urate synthesizing capacity until 36 hours before ecdysis. However, the accumulation of urate with this elevation in

in vitro synthesizing capacity occurred at the 'normal' rate. From this study it is proposed that during the LPT fat body urate storage is regulated by the availability of precursors and not via changes in urate synthesizing capacity elicited by ecdysone.

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APPENDIX

APPENDIX

METHODS FOR THE ANALYSIS OF URIC ACID

Introduction

Continuation of an investigation into the control of uric acid synthesis is *Manduca sexta* necessitated development of methods for the determination of the uric acid content of the various tissues as well as development of an *in vitro* assay to measure the uric acid synthesizing capacity of these tissues.

The first published report on the presence of uric acid in an insect tissue was in 1856 when Fabre (cited in Kilby, 1963) showed that there were particles in the fat body of some insects which gave the murexide reaction. Various methods have since been developed to quantitate the uric acid content in tissue and blood (Kern and Stransky, 1937; Kalckar, 1947; Archibald, 1957; Henry, *et al.*, 1957; McEnroe and Forgash, 1957, 1958; Liddle, *et al.*, 1959; Rice and Grogan, 1962; Tojo and Hirano, 1966; Appelt and Cvancara, 1968; Richterick, 1969; Searcy, 1969; Jung and Pareth, 1970; Tietz, 1970; Carroll, *et al.*, 1971; and Levenbook, *et al.*, 1971). Each of these procedures was designed for a particular system and, unfortunately, rather considerable modifications must be made in order to use a method designed for use with one tissue, on another.

Uric acid has been analyzed by several methods. (1) A photometric-colorometric estimation of uric acid in serum can be made by measuring the reduction of phosphomolybdic acid to molybdenum blue, accompanied by the simultaneous oxidation of uric acid to allantoin

and carbon dioxide (Offer, 1894, modified by Benedict and Hitchcock, 1915). According to Richterick (1969) use of this method has some serious flaws: (a) the Lambert-Beer law does not apply (i.e., absorption is not proportional to concentration); (b) the final solutions are turbid, and (c) the reaction is nonspecific, positive results being given by compounds such as glutathione, ergothioneine, cysteine, cystine, and caffine. (2) Procedures which involve the extraction and purification of uric acid from tissues have been employed (McEnroe and Forgash, 1957, 1958; Tojo and Hirano, 1966; and Levenbook, et al., 1971). (3) The enzymatic degradation of uric acid with uricase following the procedures of Bulgar and Johns (1941) with modifications by Kalckar (1947) and Praetorius (1949) is the most widely used. The enzymatic degradation of uric acid takes advantage of the fact that in alkaline solutions, fully ionized uric acid absorbs ultraviolet light at 293 nm. Uric acid is degraded by uricase into allantoin, carbon dioxide and hydrogen peroxide, products which do not absorb strongly at 293 nm. Further, this quick and extremely specific enzymatic-ultraviolet-photometric procedure can be used without deproteinization in contrast to the other methods which required it (Praetorius, 1949). The quantity of urate in hornworm tissues was thus determined by the enzymatic ultraviolet photometric procedure for the reasons elaborated above.

A method for determining the rate of tissue urate synthesis in vitro was also needed. Certain conditions were required for the assay in order to exclude competing reactions: a) the precursor would enter the tissues of interest from the incubation media, b) the labeled

portion of the precursor should be totally incorporated into the product, and c) that precursor should be incorporated only into uric acid, and d) uric acid and the precursor should be readily separable. The precursor chosen, formate, fulfills all of these requirements. Formate was shown to be incorporated both *in vitro* and *in vivo* into carbons 2 and 8 of the purine ring of uric acid in vertebrates and yeast (Buchanan, 1951), and in the cockroach, *Periplaneta americana* (McEnroe and Forgash, 1957, 1958) and the blood sucking bug, *Rhodnius prolixus* (Barrett and Friend, 1970) (see Figure 8). Their results refuted the theory that in insects uric acid is formed only as an end

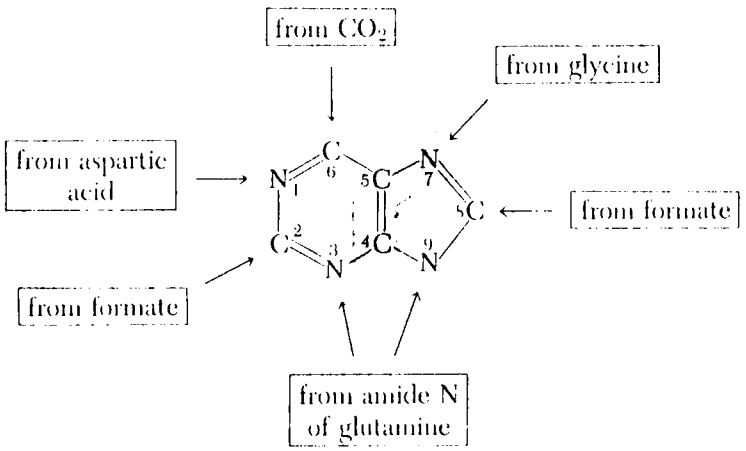

Figure 8. Origin of the atoms of the purine ring

product of nucleic acid metabolism (via the uricolytic pathway), and demonstrated that uric acid is synthesized via the uricotelic pathway following purine synthesis.

In purine synthesis, carbon 8, contributed by formic acid, is the third of the 5 carbons added (Figure 8) while carbon 2, (also derived from formic acid) is the last atom added. Thus, addition of a formyl group is the completing step in the biosynthesis of the purine ring. (The formyl group also enters fewer additional metabolic pathways than do the other ring precursors.). Clearly, purines are the precursors of uric acid whether formed by the uricolytic of uricotelic pathways. Use of labeled formate in the determination of the origin of the purine ringe and its subsequent conversion to uric acid makes formate an appealing precursor in the study of uric acid synthesis. A method for separating the ^{14}C-formate incorporated into urate from unincorporated ^{14}C-formate precursor was needed, if tissue urate synthesizing capacity was to be determined.

Materials and Methods

Animals. Animals were maintained according to the description in Chapter 1.

Quantitation of Tissue Uric Acid

Establishment of a Comparative Standard. The earlier enzymatic photometric methods of uric acid analysis were modified by Appelt and Cyancara (1968) so that these techniques could be used with tissue homogenates. This method was applied to the system for the analysis of uric acid in fat body tissue. Using as the maximum quantity of uric acid likely to be encountered in any one tissue, as the total quantity of uric acid present in intact tobacco hornworms on the day of the larval-pupal ecdysis, (Levenbook, et al., 1971) the following control experiments were performed.

If the maximum amount of urate found in an animal during the LPT is 57 mg (Levenbook, et al., 1971) and assuming a weight of 0.6 g fat body in this animal (see Chapter I), then one could expect to find 9.5 mg uric acid per 100 mg of fat body tissue. To simulate tissue assay conditions, 9.5 mg of uric acid was added to 3 ml of chilled ($4^{o}C$) 1% Li_2CO_3 and this solution sonicated at 20 second intervals for 1 minute. All of the uric acid could be solubilized in this volume of Li_2CO_3. This uric acid preparation served as the working solution throughout the control experiments. The assay procedure was identical to that described by Appelt and Cvancara with the following exceptions: (1) bovine serum albumin (Sigma Type V)was added to simulate the effect of cellular proteins present in the tissue homogenate on the action of the enzyme; (2) the effects of changes in pH or ionic strength of the Li_2CO_3 on the enzymatic activity were determined, and (3) the effects of temperature on rate of reaction was measured.

An initial attempt was made to use the Appelt and Cvancara procedure, modified by Jungreis and Tojo (1973) but it proved to be too unreliable and non-reproducbile to warrant further consideration.

A urate standard curve was prepared by using dilutions of a uric acid stock solution (1mg/ml in 1% Li_2CO_3 at $60^{o}C$) in concentrations ranging from 5×10^{-4} mg/ml uric acid to 5×10^{-2} mg/ml in 0.8% Li_2CO_3. The samples were read on a Gilford Model 252 spectrophotometer using 1% Li_2CO_3 as a blank.

Quantitation Modification I. (QMI) The assay included a blank, an unknown, and a zero time control, with a minimum of two samples at each time point. A quantity of the working urate solution (equal to the larval and pupal quantities of uric acid) was placed into each tube along with 2.5 ml 1% Li_2CO_3 (final conc = 0.83%), and 0.5 ml 0.05 M Tris-HCl (final conc = 0.008 M) buffer at pH 9.4. One milliter of uricase (0.03 units/ml of bovine kidney Type II, Sigma, dissolved in chilled (4°C) 0.05 M Tris-HCl buffer, pH 8.5, and stored at -20°C) was added to the unknowns, the samples vortexed, and incubated at 45°C for 2 hours. Nonenzymatic controls were prepared by adding 2.35 ml of 10% TCA to incubates, which were then centrifuged at 2000 x g for 30 minutes in a Sorvall Model RC2B centrifuge. At the end of the respective incubation periods, 4 ml of 10% TCA was added to blanks and unknowns, the samples centrifuged, and along with the zero time controls the absorbances were determined, as were the zero time controls. (One ml of the uricase solution was also added to the blanks following incubation.) The concentration of urate in the samples was then determined by comparing the absorbance readings against the urate standard curve.

Quantitation Modification II. (QM II) Procedures were identical to those described in QM I with the exception that incubation was for 24 hours rather than 2 hours. (See also QM IV).

Quantitation Modification III. (QM III) To determine whether uric acid would co-precipitate with protein following addition of TCA, 4 µg of bovein serum albumin (Sigma Type V, 1 mg/ml of 50 mM Tris-HCl buffer, pH 8.5) was added to each incubate. In a concurrent series of

experiments, glass test tubes were siliconized prior to incubation to determine if a portion of the uric acid would adhere to the glass.

Quantitation Modification IV. (QM IV) Procedures were identical to those in QM I except that the incubation was for 8 hours at temperatures of 30°, 37° and 45°C.

Quantitation Modification V. (QM V) Procedures were identical to those of QM I except that the effects of pH on the rate of uric acid hydrolysis were studied. It was noted that uric acid is as soluble in lithium carbonate solutions at pH 7.8 as at pH 11.0. The pH of both the lithium carbonate and Tris-buffer were then adjusted and incubation carried out at pH 7.8, 8.5 and 9.7.

Quantitation Modification VI. (QM VI) Procedures were identical to QM I except that the effects of incubation temperature between 27-45°C was studied when the assay pH was 8.5.

Chromatographic Separation of Uric Acid from ^{14}C-formate. Thin layer chromatography (see Stahl, 1969) was employed to separate ^{14}C-formate from ^{14}C-urate. First it was necessary to define a solvent mixture that would resolve uric acid as a well defined entity.

A small number of solvent mixtures employed in the chromatography of uric acid have been described: chloroform: acetone: methanol (1:1:1, v;v;v) (Rink and Gehl, 1966); acetone: chloroform: n-butanol: 25% ammonium hydroxide (30:30:40:10, v:v:v:v) (Stahl, 1969); and butanol: HOAc: H_2O, (2:1:1,v:v:v) and n-butanol: 10% formic acid (9:1,v:v) (solvent mixtures reported in literature from ICN Pharmaceuticals, 1975). The primary concern was whether these solvents could resolve uric acid and effect a separation of urate from formate. Activated polysilicic

acid plates (Gelman ITLC Type SA) were spotted with 1 µl ^{14}C-urate (25 mCi/µmole, ICN Pharmaceuticals). Plates were initially chromatographed in $CHCl_3$: Acetone: MeOH, and the solvent front allowed to advance 10 cm. Plates were dried in air at ambient temperature, and urate distribution measured in successive 5 mm strips of the thin layer plate.

Chromatography Modification I. (CM I) Chromatography was repeated employing the solvent mixtures recommended by ICN, namely butanol: formic acid (9:1) or butanol: acetic acid : water (2:1:1).

Labeled formate (0.3 µCi of Sodium formate-^{14}C, 56 mCi/mmole in ethanol: water 7:3, New England Nuclear) and urate (0.04 µCi) were spotted on plates and chromotographed as described above.

Chromatography Modification II. (CM II) Trailing of uric acid was curtailed when the solvent was butanol: acetic acid : water (2:1:1). However, good separation of formate from urate was not achieved. Therefore, the proportions of the components in the solvent mixture were systematically varied. Whatman filter paper discs were spotted with 100 µl of formate and/or urate solutions (2 ml 100% ethanol, 2 ml urate stock solution, 1 mg/ml, containing 0.3 µCi ^{14}C-formate or 0.04 µCi of ^{14}C-urate), dried under a heat lamp, placed in a scintered glass funnel connected to a vacuum line, and washed at a rate of two drops/second for 90 seconds with the following solvent mixtures: (a) butanol: acetic acid: water (2:1:1); (b) acetic acid: water (1:1); and 4 combinations of acetic acid: butanol (c) (1:1); (d) (1:2); (e) (1:3); and (f) (2:1). Then the discs were dried under a heat lamp and counted.

Chromatography Modification III. To determine what effect, if any, heat had on the retention of formate, Whatman 2 cm DEAE filter discs (Type DE 81) were spotted with each isotopic solution (see CM II) and either heat dried or air dried with subsequent heat treatment. Discs were dried for 4, 10 and 30 minutes under a lamp at approximately 60°C, with control discs dried in air then subjected to 30 mintues of heat at 60°C.

Chromatography Modification IV. (CM IV) Binding of uric acid to the DEAE discs was studied. Varying quantities of uric acid were applied to the discs. Each solution with a volume of 4 ml contained 0.04 µCi ^{14}C-urate, with 1 ml of unlabeled urate solution and 3 ml of 100% ethanol. An additional control contained 0.25 mg urate/ml water. The quantity of unlabeled urate added ranged from 0.25 mg to 1.0 mg. One hundred µl samples of the previous solution were taken. The entire sample (100 µl) of one group was spotted under vacuum while in the other group each 100 µl sample was spotted, 20 µl at a time, and allowed to absorb slowly into the disc until dry. Both groups were then washed with butanol: acetic acid (1:1) under vacuum until dry.

Chromatography Modification V. (CM V) Differential retention of formate and urate on the DEAE filter discs was studied as a function of the volume of butanol: acetic acid washing the discs. Two concentrations of cold urate, 0.05 mg/ml and 0.1 mg/ml containing 0.04 µCi ^{14}C-urate were spotted on the discs. Discs were washed under vacuum with 90-450 drops of butanol; acetic acid (1:1), followed by vacuum drying.

Chromatography Modification VI. (CM VI) The "age" of the solvent seemed to effect the differential retention of formate and urate on the DEAE filters. Butanol: acetic acid (1:1) was employed, one mixture used immediately after preparation, the second following one month in contant darkness at ambient temperature. DEAE discs were spotted with 100 µl of the respective solutions in simulated incubation medium, and washed with 450 drops of solvent under vacuum.

Chromatography Modification VII. (CM VII) The presence of acetic acid in the solvent mixture precluded determination of the quantity of butyl acetate in the older solvent mixture by mass spectrometery or gas chromatography. Consequently, the correct proportion of butyl acetate needed to selectively remove the formate while simultaneously retaining the urate, was determined empirically. Activated siliac acid thin layer plates were spotted with the isotopes and chromatographed in differing combinations of butanol: acetic acid : butyl acetate (4.5;4.5:1, 4:4:2; 3:3:4; 2.5:2.5:5).

Chromatography Modification VIII. (CM VIII) To determine those factors responsible for the loss of the formate, eight $1cm^2$ pieces of siliac acid plates were spotted with 1 µl samples of ^{14}C-urate and ^{14}C-formate. Upon spotting the isotope, 5 µl of 20% TCA was immediately added to the same spot. The eight samples were treated as follows: two were air dried, three were dried under a lamp at $57^{o}C$ and three were placed in an oven at $109^{o}C$. Each piece of plate was treated for 30 minutes.

Results

Quantitation of Uric Acid. The urate standard curve is linear to concentrations of 0.39 mg/ml uric acid (Figure 9). When the period of incubation is only 2 hours as specified by Appelt and Cvancara (1968) (QM I) only 11% of the pupal quantity and 45% of the larval quantity of uric acid (as determined by Levenbook, et al., 1971) could be recovered (Table VIII). When expressed as the absolute quantity recovered, twice as much uric acid was recovered at the higher concentration. The increase in recovery may reflect conditions wherein substrate saturation of the enzyme was not achieved.

Only 45% of the minimal quantity of uric acid found in the tissues could be recovered following 2 hours of incubation (QM I). A similar quantity was present following 24 hour incubation at 45°C and pH 9.7 (QM II, Figure 10).

The effect of an exogenous source of protein on urate recovery was found to be negligible (QM III; Table IX). However, siliconizing the tubes did increase the urate recovery from 9% to 13%. Results of QM II revealed that increasing the duration of incubation from 6-24 hours was without effect on urate recovery. Consequently, the assay was carried out under a variety of temperature and pH regimens. The percentage of urate recovery increased from 44 to 60% when the temperature was dropped from 45 to 37°C (Figure 11). However, 40% of the initial urate present was still not hydrolyzed by the uricase.

When the pH of the assay was dropped from the 9.7 value suggested by Appelt and Cvancara (1969) to pH 8.5 (QM V) a highly significant increase in urate recovery is achieved (47% → 72%) after 8 hours of incubation (Figure 12). (The enzyme may have been denatured

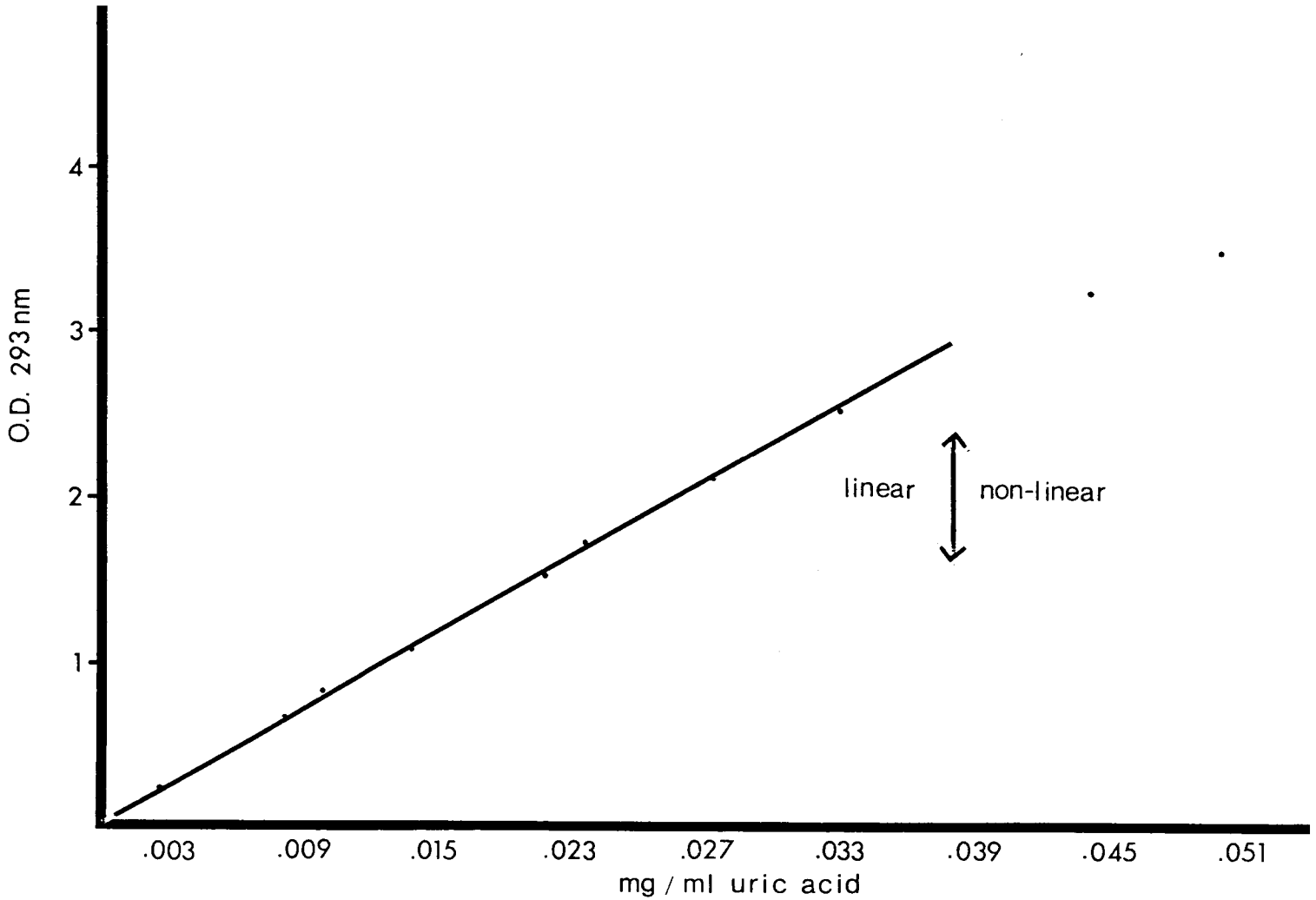

Figure 9. Uric acid standard curve

TABLE VIII

URATE ASSAY AT 45°C, pH 9.2, AND 2 HOURS INCUBATION

Stage	(N*)	Initial Concentration**	% Recovered	Amount Recovered***
Larvae	(4)	.068	45.6	.031 ± .005
Pupae	(4)	.63	11.3	.071 ± .01

*N = number separate determinations
**Initial concentrations according to Levenbook, et al., (1971) under our assay conditions
***mg ± S.E. of the mean

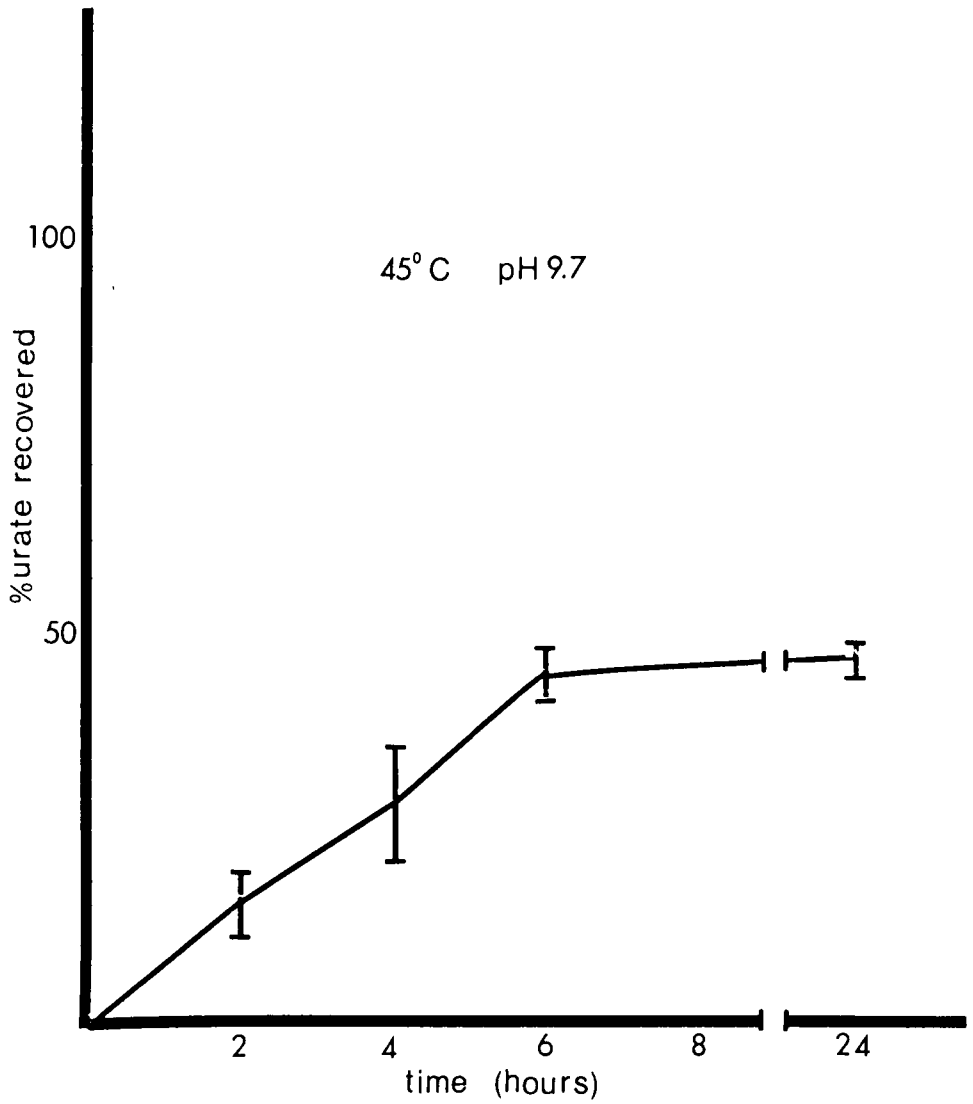

Figure 10. Urate recovery at pH 9.7 and 45°C with 24 hour incubation. Percent recovery of total urate added to uricase degradation assay procedure of Appelt and Cvancara, 1968. Percentage ± standard error of the mean.

TABLE IX

ACCURACY OF URATE ASSAY UNDER VARYING TREATMENTS

Treatment	Initial amount (mg)***	% Recovered	Absolute amount (mg)**
siliconized	.63 mg	13.7	.086 ± .003
non-siliconized	.63	9.5	.06 ± .006
S+ BSA*	.63	12.7	.080 ± .001
NS+ BSA (NS)**	.63	8.8	.056 ± .004

* S-siliconized
**NS-non-siliconized
***Mean ± S.E.

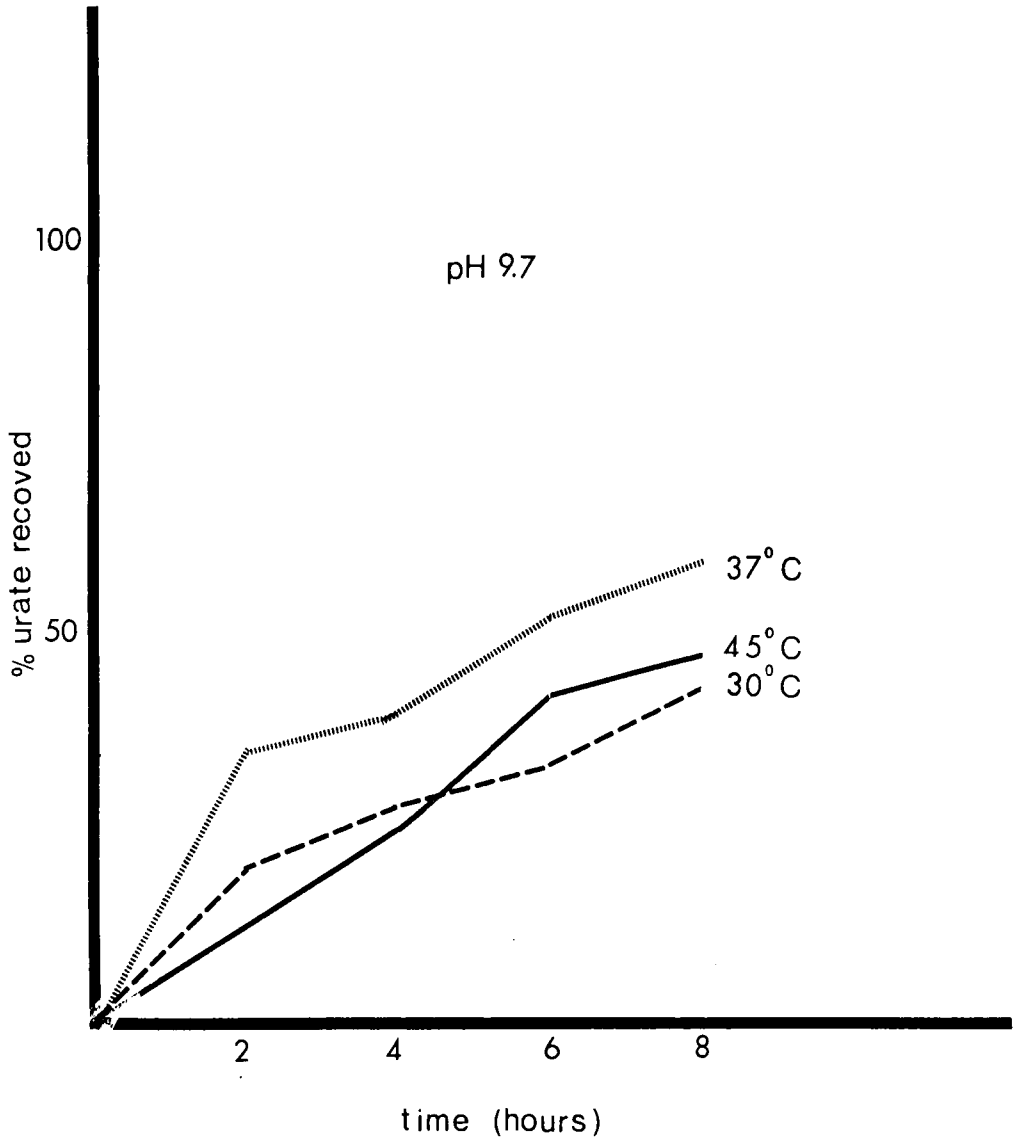

Figure 11. Urate recovery with uricase degradation at pH 9.7, varying temperature. (Less than a 2% variation around the mean in duplicate samples.)

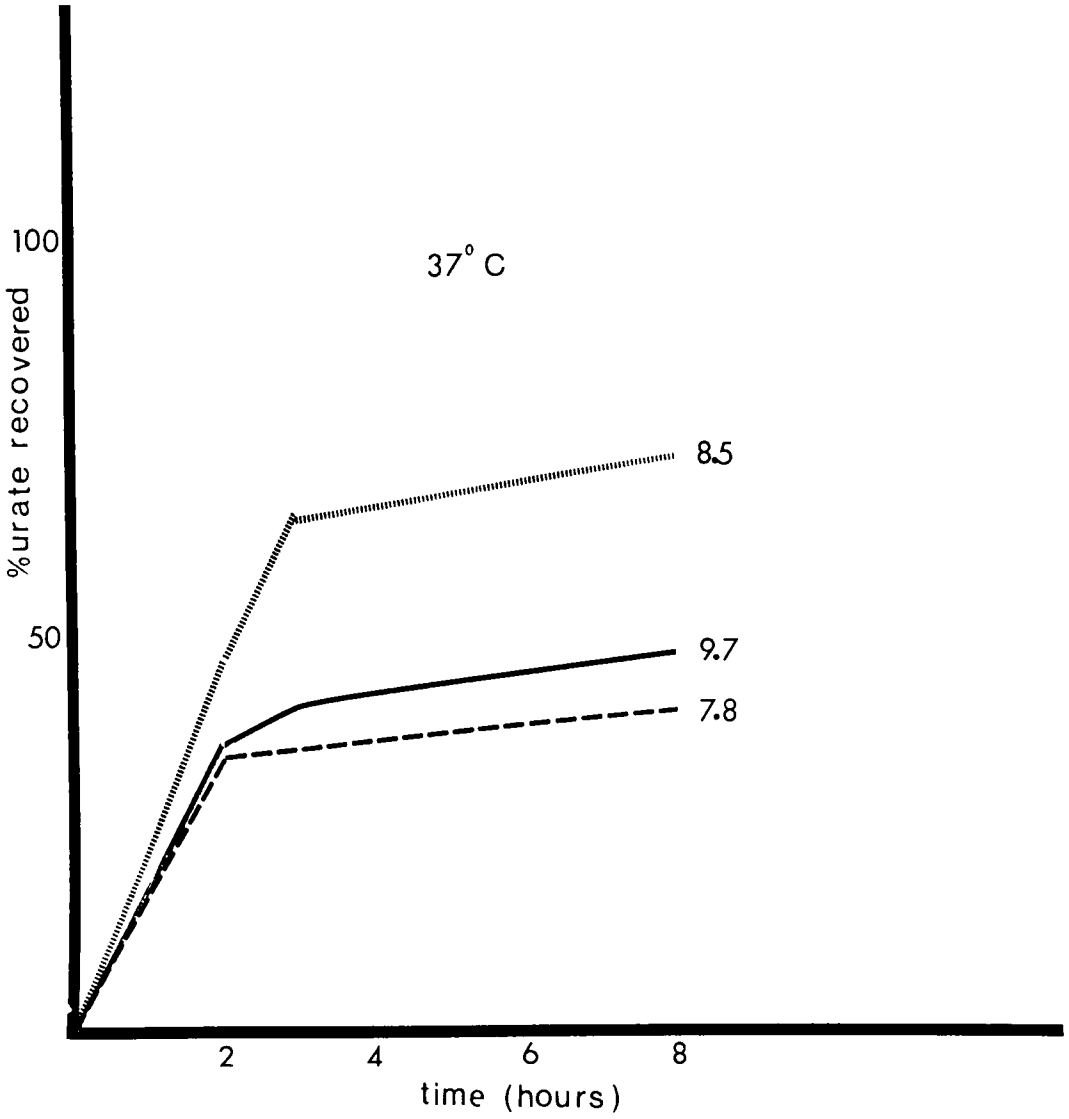

Figure 12. Urate recovery at 37°C as a function of pH. The variation around each point is less than 2%.

at the higher temperature). When the effects of temperature on urate recovery were re-examined at pH 8.5 (QM VI), up to 96% of the urate was recovered at 37°C, contrasting with 66% at 45°C (Figure 13).

Chromatographic Separation of Uric Acid from Formate: Thin Layer Plates. The profile of the resolution of uric acid by thin layer chromatography employing a variety of solvents is shown in Figure 14 (CM I). Uric acid is poorly soluble in $CHCl_3$; acetone: MeOH, and $CHCl_3$; acetone: n-butanol: 25% NH_4OH, as evidenc-d by retention of much of the urate at the origin. While uric acid was more soluble in n-butanol: 10% NCOOH, little resolution of the migrated urate was achieved (Figure 14). An alternate solvent mixture capable of separating formate from urate was therefore sought.

Butanol: 10% formic acid caused a great amount of trailing and left the majority of the labeled urate at both the origin and the solvent front. It also failed to resolve formate, since this compound was also found at the origin and solvent front (Figure 15). When n-butanol: HoAC; H_2O was employed, 95% of the formate diffused into the solvent (Figure 15) with the remainder migrating with the solvent front. With the same solvent(B) there was no trailing of the urate but it was again found at the origin and solvent front.

Chromotography separation of Uric Acid from Formate: DEAE Filter Discs. Whenthe solvent system, n-butanol: HOAc : H_2O (2:1:1), was employed (CM II), 40% of the urate and 100% of the formate were removed (Table X). Only small quantities of urate and formate were retained in HOAc : H_2O. When n-butanol: HOAc in various ratios was employed, 100% of the urate but only 20-60% of the formate was retained. Butanol:

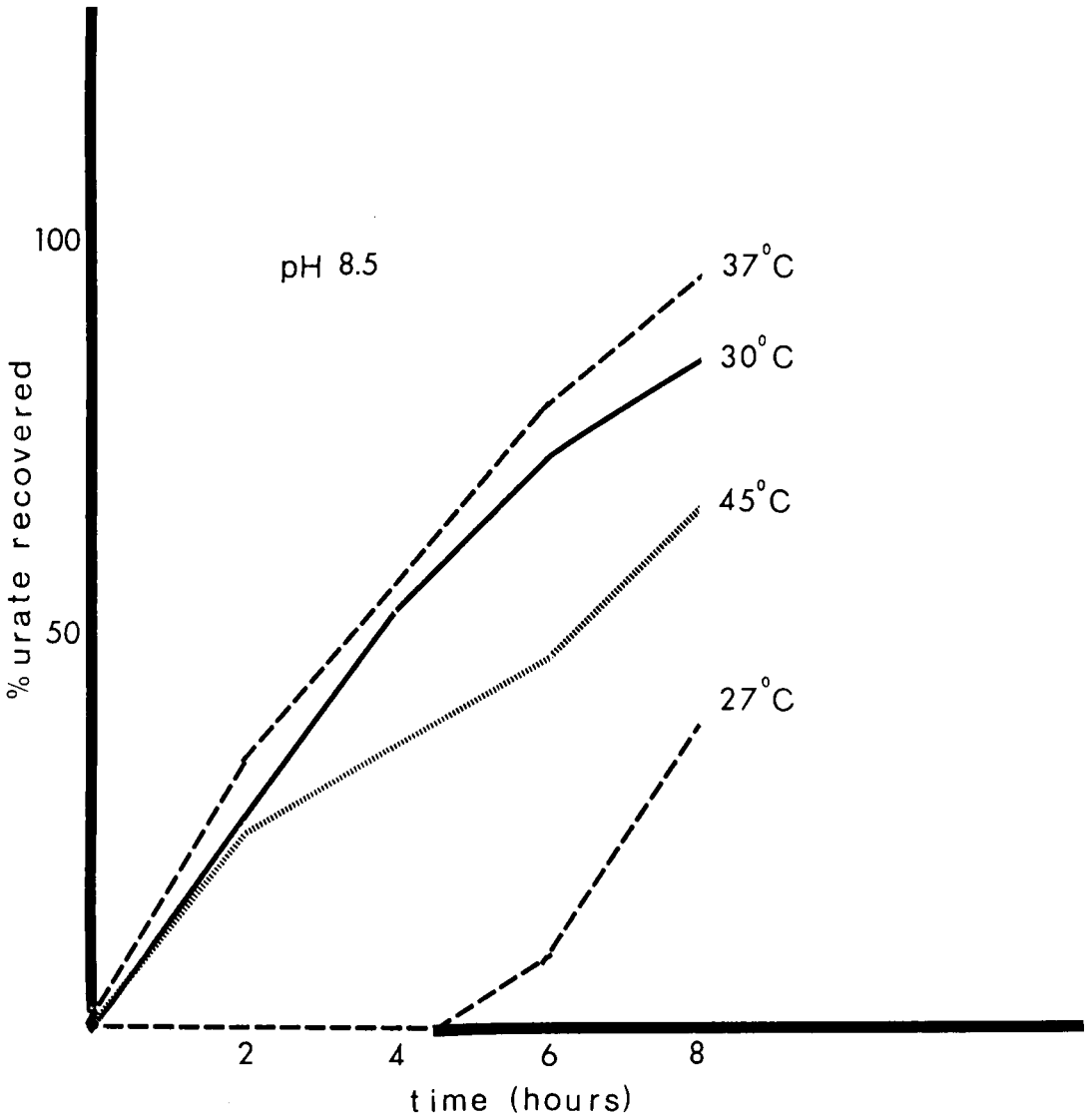

Figure 13. Urate recovery at pH 8.5 as a function of temperature. The variation around each point is less than 2%.

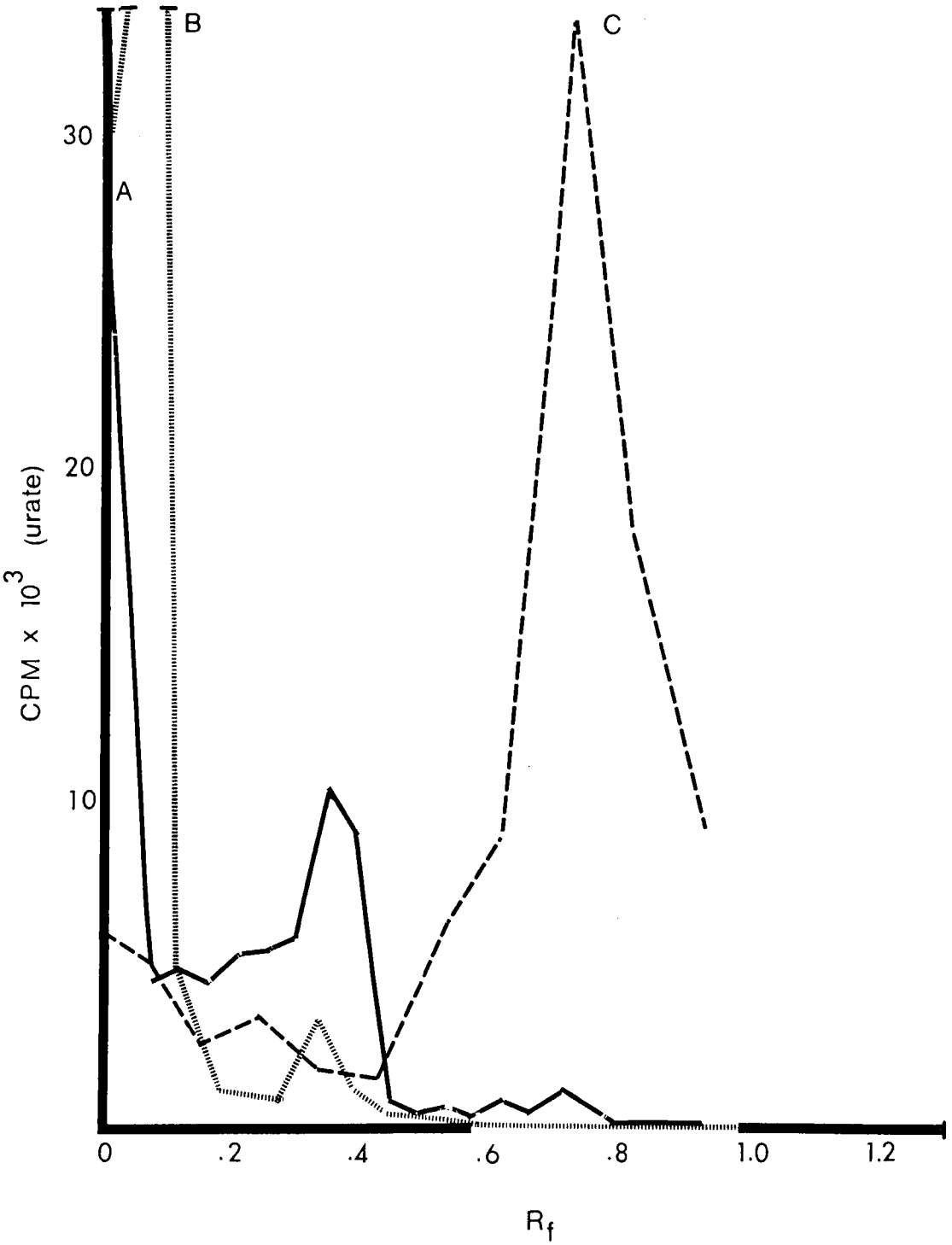

Figure 14. Uric acid thin layer chromatograph. Solvents: (a) Chloroform: acetone:methanol (30:30:30); (b) Acetone:chloroform: n-butanol: 25% ammonium hudroxide (30:30:40:10); and (c) butanol: 10% formic acid.

Figure 15. Chromatograph of uric and formic acids. (A) urate with solvent butanol: 10% formic acid, (B) urate with solvent butanol: acetic acid: water (2:1:1) (C) formate with the same solvent as (B).

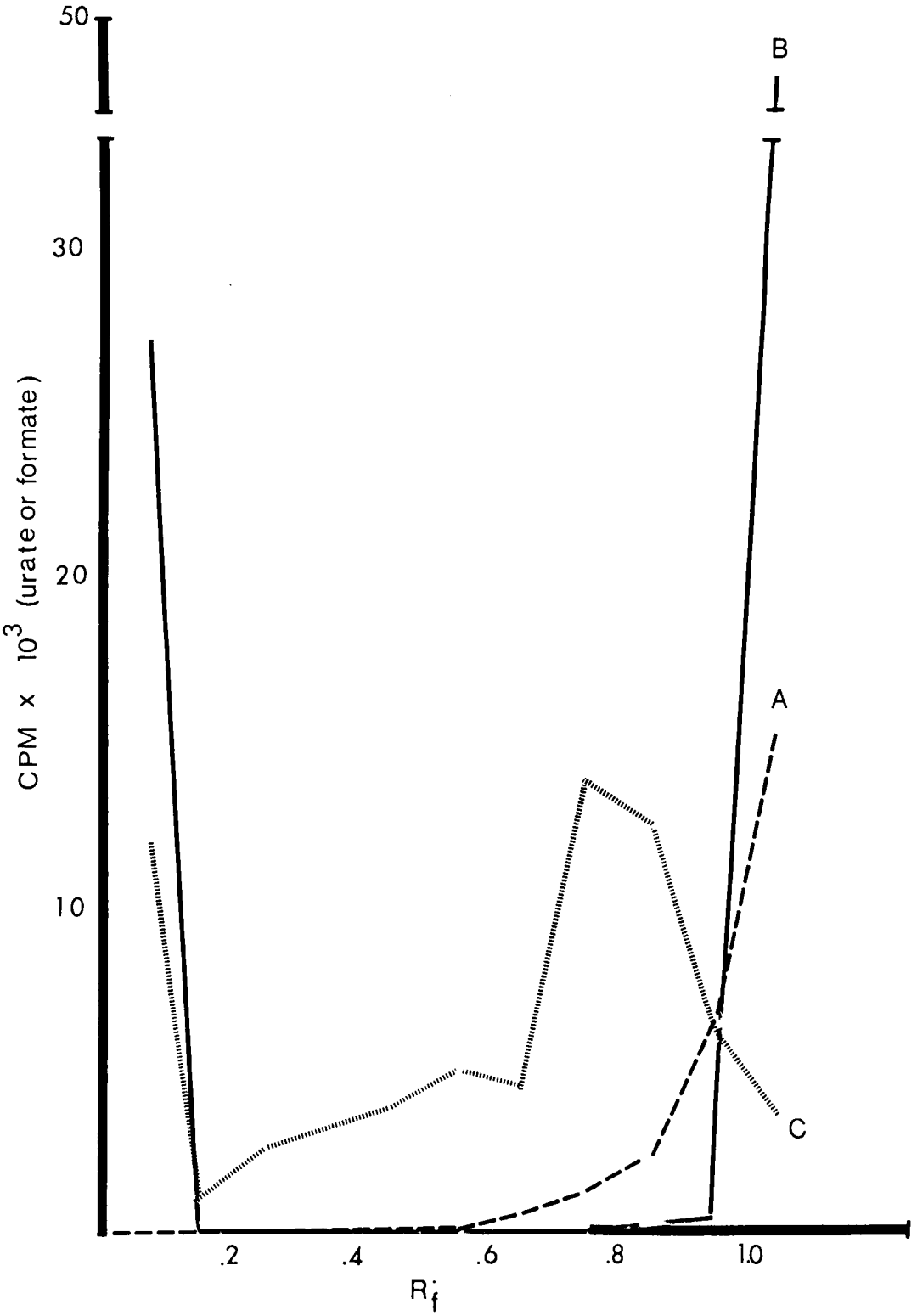

Figure 15

HOAc in a proportion of 1:1 gave the best resolution of formate from urate (Table X).

TABLE X

EFFECT OF SOLVENT PROPORTIONS ON LABEL RETENTION

Solvent	Percentage retained* Urate	Formate
a) Butanol: acetic acid: water (2:1:1)	57.95 ± 1.05	0
b) Acetic acid: water (1:1)	23.25 ± 0.85	1.0 ± 0.007
c) Acetic acid: butanol (1:2)	93.55 ± 5.75	43.2 ± 3.4
d) Acetic acid: butanol (1:3)	>100	60.9 ± 2.25
e) Acetic acid: butanol (2:1)	98.25 ± 1.75	26.1 ± 4.5
f) Acetic acid: butanol (1:1)	>100	19.3 ± 2.6

*Mean ± S.E.

In the experiments in Table X, discs were either dried in air or rapidly under a lamp (65°C). The effect of the method of drying the discs on formate/urate retention was investigated (CM III). Urate retention was not affected by heat treatment, whereas formate was affected with exposures of 30 minutes, causing a 25-100% loss (Table XI). Unfortunately the variability of retention was too great to rely upon this method.

TABLE XI

EFFECT OF HEAT EXPOSURE ON ISOTOPE RETENTION

Exposure time (minutes)	% Retention-Wet		% Retention-pre-dry	
	Formate	Urate	Formate	Urate
0	100*	100	100	100
4	100	100	100	100
10	98-100	100	100	100
20	75-78	100	100	100
30	0-74	100	100	100

*With less than 1% variation around each point.

The effects of exogenous substrate on formate and urate retention was studied (CM IV). As the quantity of unlabeled urate increases from 0 to 1:0 mg/ml, urate retention increases non-linearly from 60-90% (Table XII). Further, spotting discs with 100 vs 300 µl of sample insured better recovery, although this factor can be attributed to the rate of solute movement through the filter (Columns 4 and 5, Table XII). The effect of solvent volume on solute retention (CM V) was also studied. Differences were not observed between 0.05 mg and 0.1 mg unlabeled urate (Table XIII). The volume of washing solution affected both formate and urate retention, 4.5 ml resulting in 64% urate but only 8% formate retention (Table XIII). The solvent mixtures were therefore varied to see whether better separation could be achieved.

When solvent age was considered (CM VI) it was observed that new solvent retained 74% of the urate and 37% of the formate while solvent

TABLE XII

EFFECT OF SPOTTING METHOD ON ISOTOPE RETENTION

Cold Urate mg/ml	% Retained* with Vacuum per sample size 100 µl	200 µl	300 µl	% Retained without vacuum per sample size 80 µl	100 µl
0	59	36	25	ND**	ND**
0.25	82	48	37	32***	38***
0.50	90	53	42	100	100
0.75	88	61	43	97.5	98
1.00	93	55	48	100	98.5

*Less than 2% variation occurred around each point
**ND- not determined
*** Sample is distilled water

TABLE XIII

EFFECT OF VOLUME OF SOLVENT* WASH ON ISOTOPE RETENTION

Wash Volume (ml)	% Retained** with 0.05 mg/ml cold urate	cold formate	% Retained with 0.1 mg/ml cold urate	cold formate
no wash	100	90	100	85
4.5	64.5	6	64	9.5
12.5	55.5	2.5	54	5.5
22.5	57.5	3.0	49.5	2.0

*BuOH:OHAc (1:1)
**With less than 3% variation around each point

prepared one month earlier retained 63% of the urate and 8% of the formate.

	Urate	Formate
New solvent	74.2 ± 0.2	37.65 ± 1.35
Old solvent	62.6 ± 0.6	8.0 ± 0.1

This showed that solvent "age" affects its resolving capacity with respect to formate and urate. A change in the equilibrium between n-butanol and acetic acid apparently occurs with time resulting in differing quantities of butyl-acetate.

A profile of a chromatogram demonstrating the ability of solvent mixtures of n-butanol: HOAc:Butyl-Acetate (CM VII) to resolve formate and urate is shown in Figure 16. Decreases in Rf are noted with increasing concentrations of butyl acetate. Accompanying the shift in Rf is an increase in urate retention at the origin. All of urate spotted on the plates was retained. The insert on Figure 16 shows that little of the formate is retained, and that which is retained is located within 1 cm of the origin. Solvents containing 10% or 20% butyl acetate, promote 100% retention of urate under a clean peak, while simultaneously removing 99% of the formate. In experiments measuring ^{14}C-formate incorporation into ^{14}C-urate, n-butanol: HOAc: butyl acetate in a ratio of 2:2:1 was chosen over 4.5:4.5:1 because it resolved a common contaminant present in the ^{14}C-urate.

The disappearance of the formic acid from the plates suggests migration of formate into the solvent, but the absence of isotope in the solvent revealed that it must have been volatilized. As illustrated in Table XIV formate was indeed volitalized (CM VIII) following acidification, whereas the urate was not affected. From these data the

Figure 16. Chromatograph of uric acid with the solvent n-butanol: acetic acid (1:1). With (A) 10% butyl acetate, (B) 20% butyl acetate, (C) 40% butyl acetate (D) 50% butyl acetate. The insert shows the formate retention under the same solvent regimen as the urate.

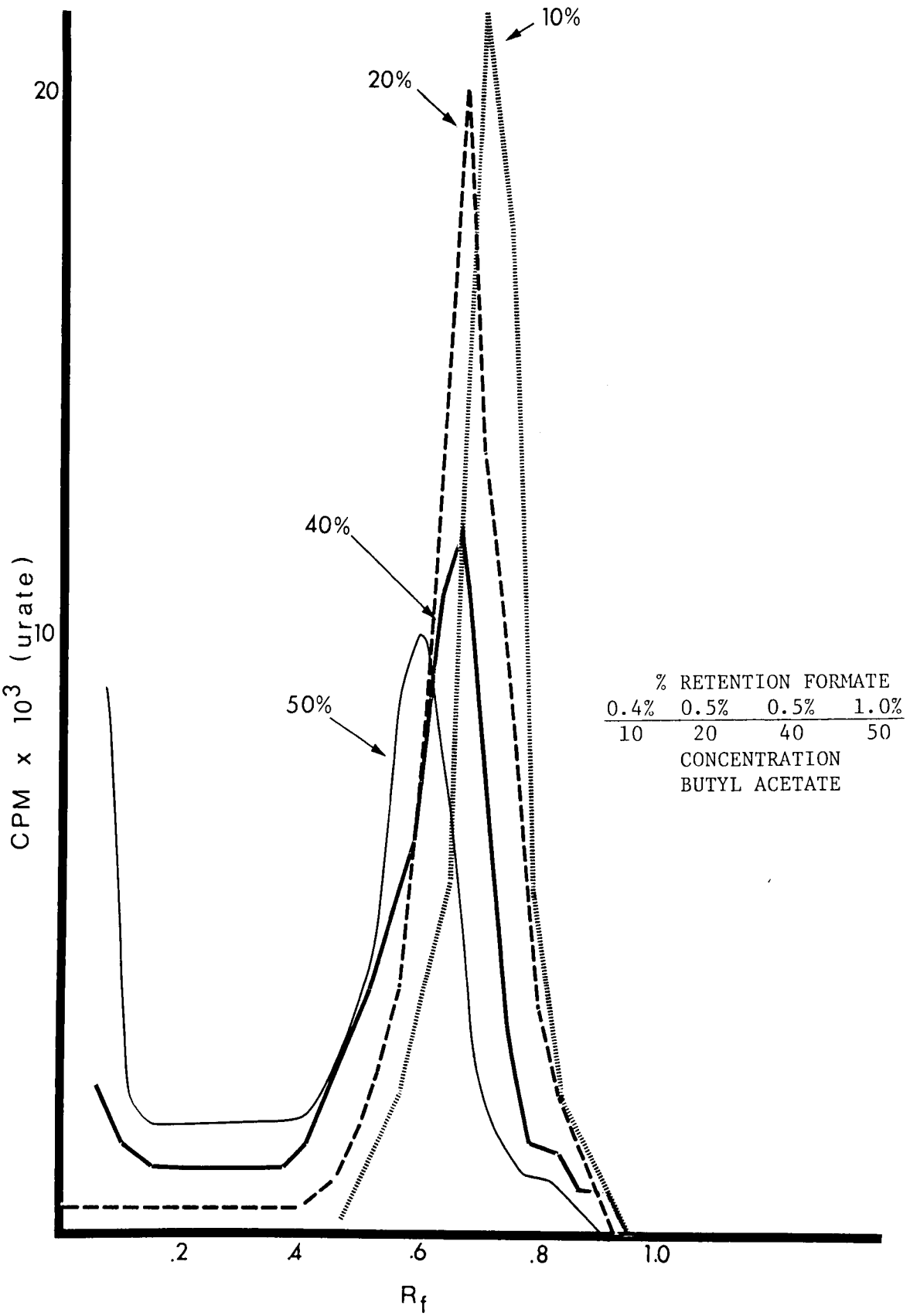
10%
20%
40%
50%
20
10
CPM x 10^3 (urate)
.2
.4
.6
.8
1.0
R_f
% RETENTION FORMATE
0.4% 0.5% 0.5% 1.0%
10 20 40 50
CONCENTRATION
BUTYL ACETATE

Figure 16

TABLE XIV

HEAT TREATMENT AFTER ACIDIFICATION OF URIC AND FORMIC ACIDS

Treatment*	% Retention ($\bar{x}$) Urate	Formate
air dried	100	8.3 ± 2.0
109° oven	95.05 ± 5.95	0.5 ± 0.1
57° lamp	91.80 ± 8.20	0.25 ± 0.05

*With trichloroacetic acid pretreatment

best method of separating formate from urate seems to be acidification followed by heat treatment, at 109°C. Dehydration of the formic acid appears to occur in the presence of trichloroacetic acid ($HCOOH \rightarrow H_2O + CO$).

Discussion

Introduced by Kalckar (1947) and Praetorius (1949) and modified by Reichtmeir and Wrenn (1955), the earliest methods of uric acid analysis employing uricase were designed for use with mammalian serum. It was not until 1968, that Appelt and Cvancara devised a method for the analysis of uric acid in tissue homogenates.

Until now, researchers in the area of insect physiology have gone to considerable effort to extract and analyze uric acid (Cole, 1944; Bursell, 1964; Tojo and Hirano, 1966; Barrett and Friend, 1970; Carroll, et al., 1971; Kageyama, 1971; Jungreis and Tojo, 1973). Modifications of the method of Appelt and Cvancara as outlined in this

Appendix have simplified and increased the efficiency of the procedure for the analysis of uric acid in tissue preparations. The advantages of these modifications are as follows. (1) The enzyme is now functioning at maximum velocity under conditions of controlled temperature and pH. (2) Extending the period of incubation to 8 hours results in greater degradation of urate. Samples can be left unattended throughout the incubation if they are slowly agitated in a water bath. (4) All assays can be run in volumes of less than 5 ml, and (5) only a small aliquot of a well mixed incubation solution need be analyzed. These samples can them be centrifuged at 8,000 x g for 1 minute as opposed to 30 minutes at 2,000 x g.

The assay was further expedited by determining that the complete degradation of unknown uric acid tissue samples was unnecessary when dilute samples were used (i.e., those with absorbances under 1.0). With this method, the zero time controls were always at a maximum of 10% of the uricase samples. When large numbers of samples were to be run a few were placed with the uricase system and then zero time controls were taken. It representative samples of the zero time controls are within a maximum of 10% of the uricase samples, then the bulk of the samples do not have to be analyzed by uricase degradation. Samples of sonicates could be placed in the incubation media, vortexed, precipitated with TCA, centrifuged, and the absorbance of the supernatants recorded. Altering the pH and temperature of the assay increased the recovery of uric acid to 100%. This assay is the most efficient and reliable method for analyzing uric acid in crude insect tissues, since fewer reagents, manipulations, and less sample are needed.

McEnroe and Forgash (1957,1958) and Barrett and Friend (1970) have shown that ^{14}C-formate could be recovered either *in vivo* of *in vitro*, respectively, in carbons 2 and 8 of uric acid. Using the cockroach, *Periplaneta americana*, McEnroe and Forgash (1957) noted that only 3% of the label was recovered in uric acid, in contrast to the results of Barrett and Friend (1970) with the blood-sucking bug, *Rhodnius prolixus*, who recovered 97% of the incorporated label in carbons 2 and 8 of the purine ring. Similar studies have shown results in close agreement with those of Barrett and Friend: 92% in the cockroach (McEnroe, 1957);94.4% in the snail, *Otala lactea* (Lee and Campbell, 1965) and 96-98% in the pigeon (Karlson and Barker, 1949; Elwyn and Sprinson, 1950). McEnroe and Forgash (1958) did show through studies done *in vitro*, that 93% of the label was in carbons 2 and 8 and another 6% was in carbons 4, 5 and 6 of the purine ring.

These earlier studies laid the foundation for *in vitro* studies of uric acid synthesis in the fat body of the tobacco hornworm, *Manduca sexta*. Separation of free formate from that incorporated into urate by fat body tissue *in vitro*, is the simplest procedure for the analysis of uric acid synthesis in insect tissues. The two methods, thin layer chromatography and acidification with heat prove quite adequate for this purpose. The chromatographic method can be used to test the specificity of label incorporation, while acidification and heat treatment measure the quantity of uric acid synthesized by selectively removing all of the free formic acid, but none of the newly synthesized uric acid.

VITA

Patsy Kylene Williams Boyce was born in Burnsville, North Carolina on April 28, 1952. She received her elementary education in North Carolina and Tennessee Public Schools, and her secondary education was received in Cocke County High School, Newport, Tennessee. After graduating from high school in May, 1970, she entered Berea College, Berea, Kentucky and in May, 1974 received a Bachelor of Arts degree in Biology.

In September, 1974 she entered the Graduate School at the University of Tennessee, Knoxville. While conducting her research she was supported by a graduate teaching assistantship until August, 1976. September, 1976 she was employed and is currently working as a research assistant for the department of Zoology at the University of Tennessee, Knoxville. She graduated with a Master of Science degree in Zoology, December, 1977.

She is married to Stephen L. Boyce of Nashville, Tennessee.