ABSTRACT

Wendy E. Quinn. LEVELS OF N-ACETYLGLUCOSAMINE DURING THE MOLT CYCLE OF Callinectes sapidus Rathbun. (Under the direction of Edward P. Ryan) Department of Biology, April 1980.

The purpose of this experiment was to test the hypothesis that the content of N-acetylglucosamine varied with the molting stage of the blue crab, Callinectes sapidus Rathbun. The breakdown product of chitin, N-acetylglucosamine, was determined colorimetrically. N-acetylglucosamine levels increased in premolt and decreased after ecydsis. This would be expected as the chitin must be broken down prior to ecydsis in order to be reutilized in the formation of the new cuticle.

LEVELS OF N-ACETYLGLUCOSAMINE DURING THE MOLT CYCLE OF *Callinectes sapidus* Rathbun

A Thesis

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by

Wendy E. Quinn

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APPROVED BY:

SUPERVISOR OF THESIS

Edward P. Ryan, Ph.D.

THESIS COMMITTEE

W. James Smith, Ph.D.

Takeru Ito, Ph.D.

James M. Joyce, Ph.D.

ACTING CHAIRPERSON OF THE DEPARTMENT OF BIOLOGY

Edward P. Ryan, Ph.D.

DEAN OF THE GRADUATE SCHOOL

Joseph G. Boyette, Ph.D.

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INTRODUCTION

One of the characteristics common to the members of the phylum Arthropoda is the process of molting. In crustaceans, molting is a continuous cycle, necessary for growth from first to final instar. Molting is cyclic and was divided into various stages by several workers (1,2,3). Drach established the most commonly used classification system for the molting stages. Baumberger and Olmsted used physical characteristics to stage crabs into six categories (3). These divisions were: 1) hard, 2) pillan or peeler, 3) about to molt, 4) newly molted, 5) soft, 6) paper shell. Passano's classification modified Drach's somewhat and included five major stages with substages in all but the molt stage (Table 1). His criteria were primarily based on cellular changes.

The five major stages were E, A, B, C, and D. Stage E was that point at which the actual molt or ecydsis occurred. Immediately following was stage A with two subdivisions, A_1 and A_2, in which the crab was newly molted and soft. This was known as early postmolt. The next stage was B, in which the crab's shell had hardened somewhat and was known as a paper shell crab. This late postmolt stage has two further divisions, B_1 and B_2. Stage C which followed B was known as the intermolt stage and had four divisions, C_1, C_2, C_3, C_4, and a possible fifth division C_4T for those crustaceans that reached permanent anecydsis. Certain species reached a particular level of growth and no longer underwent ecydsis. At stage C, the cuticle had become hard. The following stage D had four divisions, D_1, D_2, D_3, and D_4. This stage was known as premolt.

TABLE 1

MOLT STAGES

Stage	Name	Characteristics
Stage A		
A_1	Newly molted	Continued water absorption and initial mineralization
A_2	Soft	Exocuticle mineralization
Stage B	Paper shell	
B_1		Endocuticle secretion begins
B_2		Active endocuticle formation, chelae hard; tissue growth begins
Stage C	Hard	
C_1		Main tissue growth
C_2		Tissue growth continues
C_3		Completion of exoskeleton; membranous layer formed
C_4		"Intermolt"; major accumulation of organic reserves
or		
C_4T	Permanent anecdysis	Terminal stage in certain species; no further growth
Stage D	Proecydsis	
D_0		Epidermal and hepatopancreas activation
D_1		Epicuticle formed and spine formation begins
D_2	Peeler	Exocuticle secretion begins
D_3		Major portion of skeletal resorption
D_4	About to molt	Ecdysial sutures open
Stage E	Molt	Rapid water uptake and exuviation

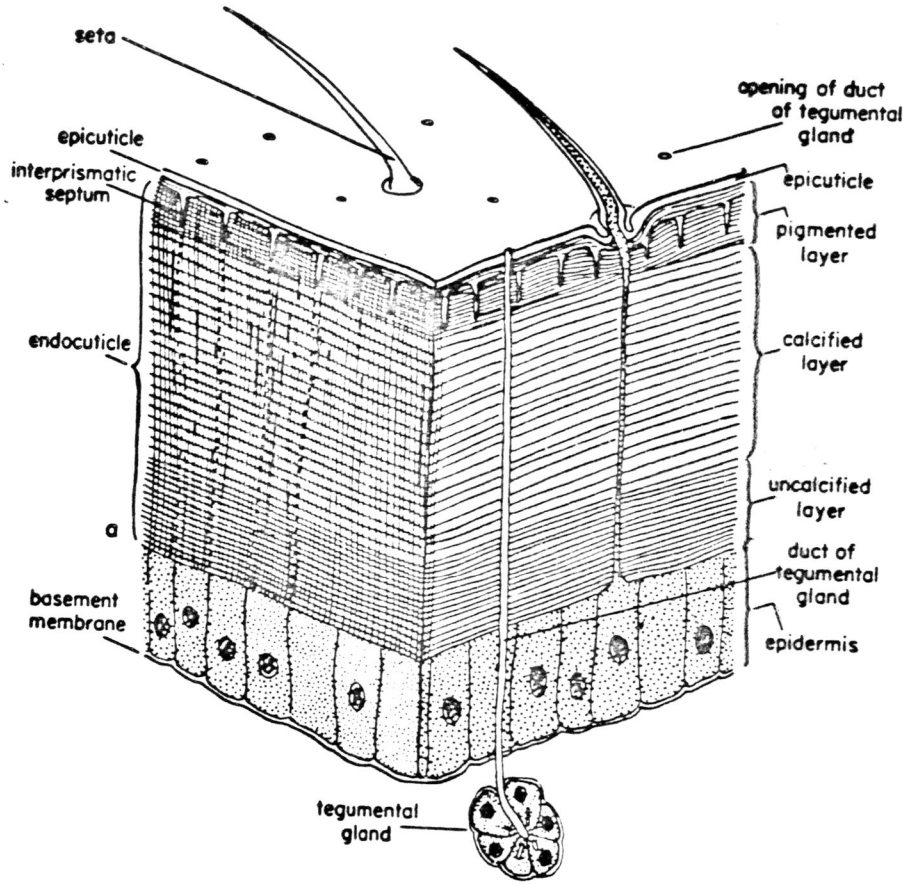
From: Passano (2)

The term apolysis designates the actual "freeing of the epidermal cells from the old exoskeleton" (4), whereas ecydsis is used to designate the actual casting off the old exoskeleton, when the arthropod emerges from the old exoskeleton (5). The exoskeleton or cuticle of the crustacean is made up of several layers, the epicuticle being the outermost layer (Figure 1). Directly underneath are: the pigmented layer, the calcified layer and the uncalcified layer; these three layers are collectively known as endocuticle, since all contain chitin. Below the endocuticle is the epidermis. Prior to ecydsis, a new endocuticle is laid down directly underneath the old cuticle. All layers with the exception of the uncalcified layer are laid down prior to ecydsis (6). The epicuticle is secreted by tegumental glands, which are below the epidermis. The epicuticle is laid down post ecydsis. Much of the chitin in the old cuticle is reabsorbed and utilized in the formation of the new cuticle (7).

During molting the old cuticle is partially digested by a chitinase. Since the endocuticle of the crab is secreted by the epidermis, it would be reasonable to assume that chitinase would be found in the epidermis. This research was conducted to test the hypothesis that the levels of chitinase would vary with the molt stage, increasing just prior to ecydsis since reabsorption of cuticle takes place at that time. The levels of endogenous N-acetylglucosamine, the breakdown product of chitinase, were measured and regarded as a measure of the activity of chitinase.

FIGURE 1

DECAPOD CUTICLE. DIAGRAM ILLUSTRATING STRUCTURE OF DECAPOD CUTICLE AS SEEN IN VERTICAL SECTION. FROM PASSONO (2).


seta
epicuticle
interprismatic septum
endocuticle
a
basement membrane
tegumental gland
opening of duct of tegumental gland
epicuticle
pigmented layer
calcified layer
uncalcified layer
duct of tegumental gland
epidermis

LITERATURE REVIEW

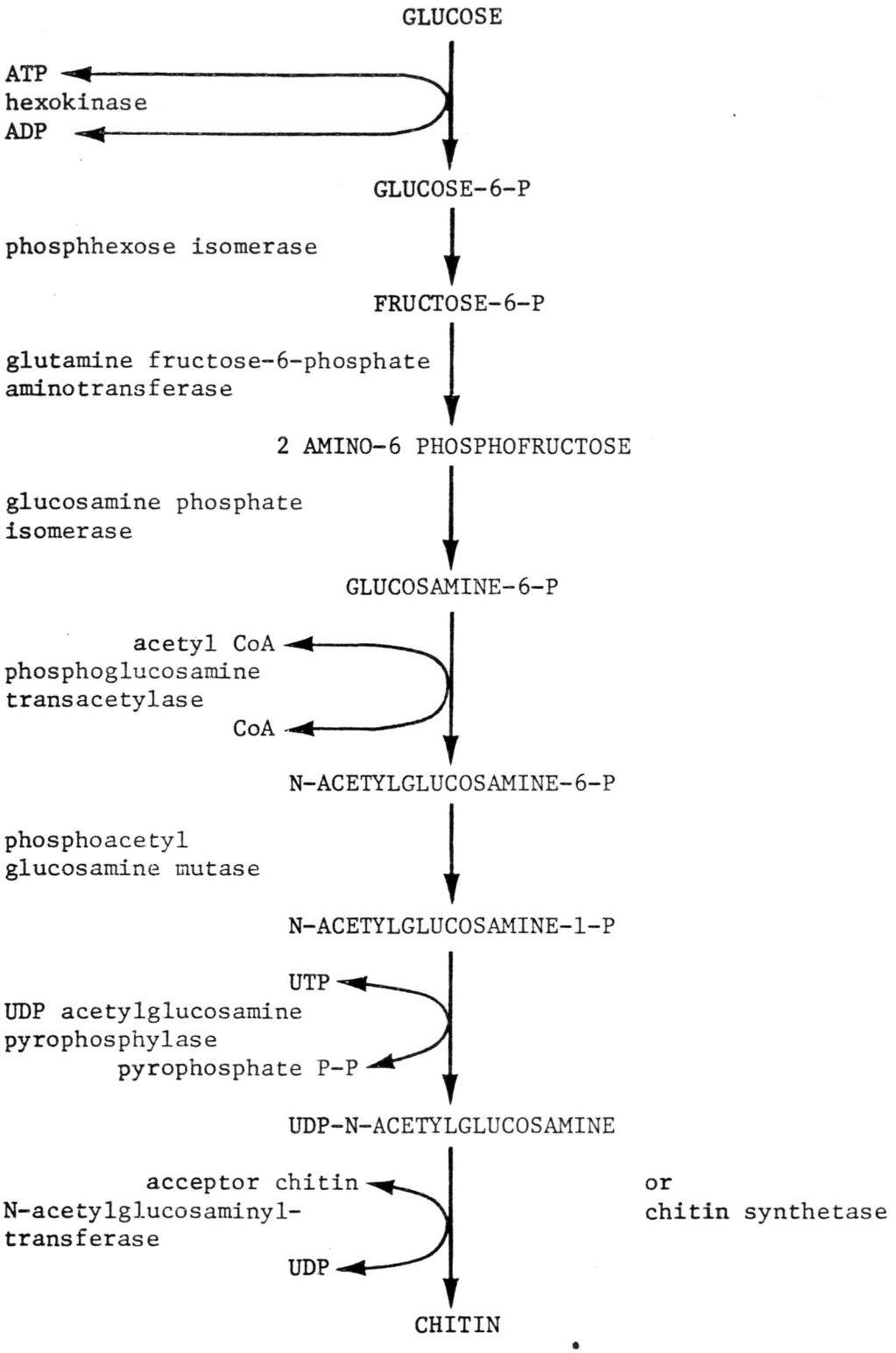
Chitin is a homopolysaccharide composed of N-acetylglucosamine in β (1→4) linkage (8). Karrer and Hofmann using snail gut enzyme established that N-acetylglucosamine was the basic unit of the polysaccharide-chitin (9). Shortly after this work, Clark and Smith did extensive work on the physical properties of chitin (10). They showed that chitin had a thin upper layer of "encrusting" material usually made up of calcium carbonate. Additionally, they recorded that the tensile strength of chitin was a remarkable 58 kg per square mm and that chitin was comprised of orthorhombic crystals. Neville et al., using the electron microscopy, showed that chitin in crustaceans was found as a crystallite, 2.8 nm across in a pseudo-hexagonal lattice (11). Immediately following ecydsis, the lattice became secondarily reoriented as the cuticle expands. Each crystallite had a maximum of six chains of poly N-acetylglucosamine per sheet with a total of three sheets.

In 1965, Carey demonstrated that labeled acetylglucosamine was incorporated into chitin by the blue crab, Callinectes sapidus (12). The synthesis of chitin was catalyzed by chitin synthetase. The majority of the work since Carey's experiments was carried out with yeast, chitin being a constituent of yeast cell walls. Work on the metabolic pathway of chitin synthesis can be carried out with greater ease in this organism. Chitin synthetase was found largely in an inactive form designated "zymogen" (13). The zymogen was activated by an "activating factor" which was a protease. Trypsin was found to act as an activator in addition to this endogenous protease (13,14,15). In addition, there

was a heat stable protein inhibitor (13,16). It was formerly believed to be an inhibitor of chitin synthetase itself but this was found not to be true (15). Chitin synthetase apparently catalyzed the synthesis of chitin by adding the N-acetylglucosamine unit of uridinediphosphate-N-acetylglucosamine (UDPAG) to a pre-existing shorter chitin (17,18,19, 20). A more accurate designation for chitin synthetase is UDP acetyl-glucosamine transferase (E.C. 2.4.1.16), proposed by Hori et al. (21). However, chitin synthetase is only one enzyme among others in a more complex biosynthetic pathway from glucose to the final product chitin. Gwinn and Stevenson briefly summarized the metabolic pathway leading to chitin in the crayfish (22,23). The pathway is given in Figure 2. The starting point of the biosynthetic pathway is glucose. Glucose is phosphorylated by a hexokinase with a concomitant conversion of ATP to ADP. The resulting glucose-6-P is converted to its isomer fructose-6-P by a phosphhexose isomerase. Surhold has found the enzyme glutamine-fructose-6-phosphate aminotransferase in the migratory locust (E.C. 2.6.1.16) which was responsible for the "de novo" synthesis of 2 amino-6 phospho-fructose from fructose-6-P (24). The next step involves the amination to form glucosamine. Gwinn and Stevenson designated this enzyme as a glucosamine phosphate isomerase. The acetylation of glucosamine to form acetylglucosamine-6-P is catalyzed by the enzyme phosphoglucosamine tranacetylase which requires acetyl CoA. The conversion of N-acetyl-glucosamine-6-P to N-acetylglucosamine-1-P is catalyzed by the enzyme phosphoacetyl glucosamine mutase (17,25). The next step links UDP to N-acetylglucosamine to form UDP-N-acetylglucosamine and is catalyzed by

FIGURE 2

FORMATION OF CHITIN FROM GLUCOSE

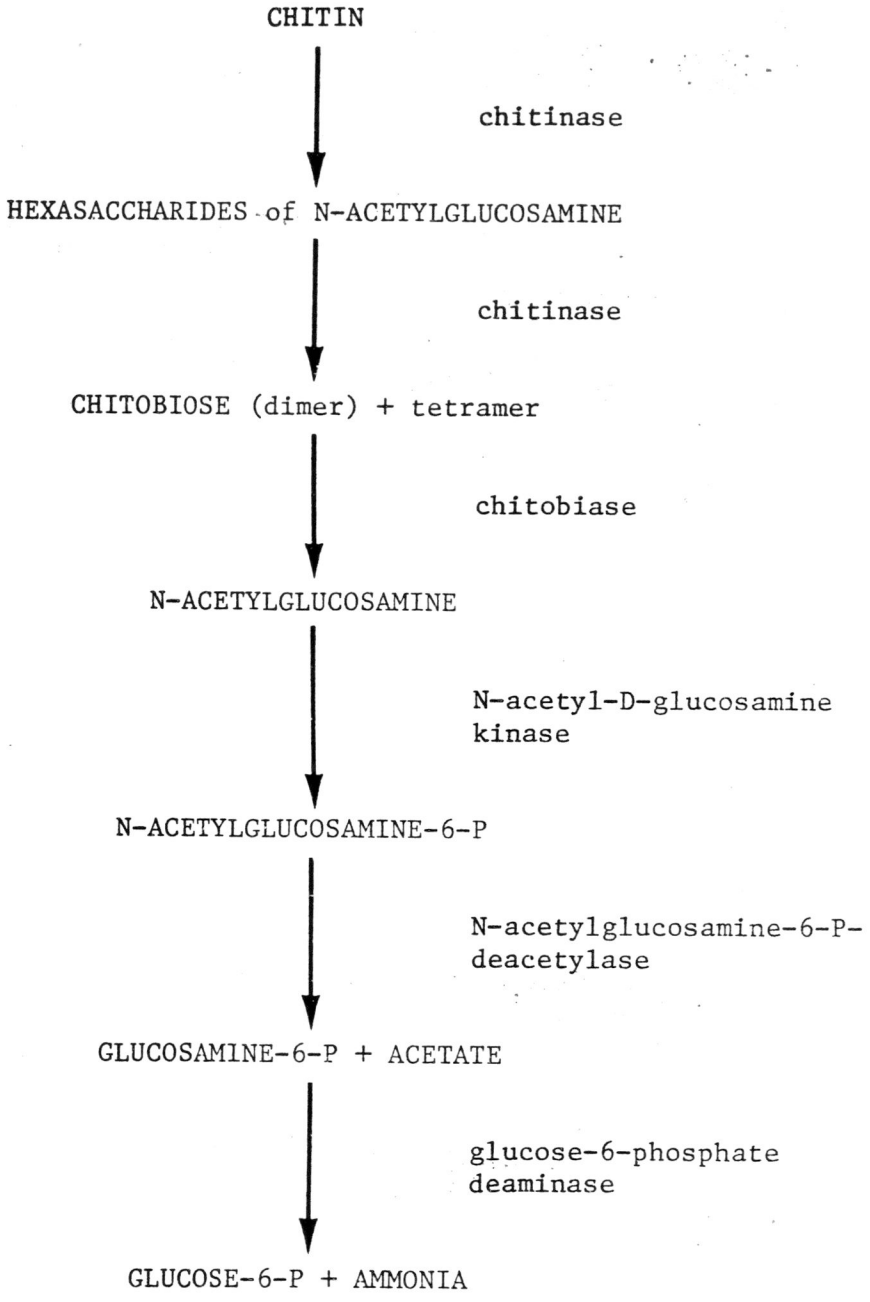

GLUCOSE
ATP
hexokinase
ADP
GLUCOSE-6-P
phosphhexose isomerase
FRUCTOSE-6-P
glutamine fructose-6-phosphate
aminotransferase
2 AMINO-6 PHOSPHOFRUCTOSE
glucosamine phosphate
isomerase
GLUCOSAMINE-6-P
acetyl CoA
phosphoglucosamine
transacetylase
CoA
N-ACETYLGLUCOSAMINE-6-P
phosphoacetyl
glucosamine mutase
N-ACETYLGLUCOSAMINE-1-P
UTP
UDP acetylglucosamine
pyrophosphylase
pyrophosphate P-P
UDP-N-ACETYLGLUCOSAMINE
acceptor chitin
N-acetylglucosaminyl-
transferase
UDP
or
chitin synthetase
CHITIN

UDP-acetylglucosamine pyrophosphorylase (17). The final step involves the addition of the monosaccharide acetylglucosamine to an endogenous acceptor molecule which forms an elongated chitin. The enzyme involved in this final step is N-acetylglucosamine transferase (E.C. 2.4.1.16), and was identified as "chitin synthetase" by Hori et al. (21). Several workers have isolated a preparation that catalyzed the transfer of acetylglucosamine from UDP-acetylglucosamine to an endogenous acceptor (26,27). This acceptor molecule accepts the N-acetylglucosamine (26,27).

The breakdown of chitin follows a different pathway than does its synthesis. The term chitinase was used very loosely by a number of workers to cover a large collection of enzymes involved in the breakdown of chitin (28,29). Chitinase activity was found in a variety of plants and animals. For example, Wargo found chitinase in the stem and root tissue of sugar maple (30). β-N-acetylglucosaminidase was found in rat lysosomes and human urine (31,32). The basic steps involved in the enzymatic lysis of chitin are summarized in Figure 3. Initially, chitin is broken down by a lysozyme-like enzyme (33). This enzyme can break down the hexasaccharide of N-acetylglucosamine at one glucosidic bond into a dimer and a tetramer (8,34). The dimer is chitobiose (34). Chitobiose or N,N-diacetylchitobiose is hydrolyzed by chitobiase (35,36). The enzyme chitobiase appears to be inducible (33). Chitobiase was found in two different forms, 1 and 2, by different workers (37,38). They had pH optima--namely, 4.5 and 6.5--and were separated by ultracentrifugation. These two chitobiase forms apparently worked in unison to hydrolyze chitobiose. In 1978, Bade hypothesized that these were two types of chitinase, one of which attached specifically to chitin (39). She

FIGURE 3

CATABOLISM OF CHITIN


CHITIN
chitinase
HEXASACCHARIDES of N-ACETYLGLUCOSAMINE
chitinase
CHITOBIOSE (dimer) + tetramer
chitobiase
N-ACETYLGLUCOSAMINE
N-acetyl-D-glucosamine kinase
N-ACETYLGLUCOSAMINE-6-P
N-acetylglucosamine-6-P-deacetylase
GLUCOSAMINE-6-P + ACETATE
glucose-6-phosphate deaminase
GLUCOSE-6-P + AMMONIA

also postulated that at the beginning of the molt cycle the chitin was inaccessible to chitinase. "The chitin is rendered competent to serve as substrate for hydrolytic degradation through attack by trypsin-like molting fluid protease" (39). This correlated well with Cabib's "zymogen and activating factor" hypothesis. "After the cuticle is rendered competent, the attacking chitinase binds to the cuticle chitin" (39).

Once chitin was broken down to N-acetylglucosamine the catabolic process was relatively simple. N-acetylglucosamine was phosphorylated by N-acetylglucosamine kinase (40). The product N-acetylglucosamine-6-P was deacetylated in the next step by N-acetylglucosamine-6-P deacetylase to form glucosamine-6-P and acetate (41). The former was deaminated by glucosamine-6-phosphate deaminase to produce glucose-6-P and ammonia. The preceding two steps were inducible according to Singh and Datta who stated that "induction of glucosamine-6-phosphate deaminase involved induced synthesis of deaminase specific MRNA and possible selective promotion of gene transcription by the inducer" (40).

The blue crab Callinectes sapidus Rathbun reabsorbs portions of its old exoskeleton in order to synthesize a new cuticle, as usually found in crustaceans (22,23). After partial reabsorption, formation of the new cuticle continues after ecydsis. It is necessary for chitin to be hydrolyzed into usable components for it to be reutilized. N-acetylglucosamine would be the most valuable form into which chitin could be hydrolyzed, since it is an obvious precursor of chitin (22,23).

MATERIALS AND METHODS

Collection and Maintenance of Specimens

Crabs were collected from the Pamlico River near Bath and Bayview, North Carolina. They were collected with a crab pot or dip net from pillings. Prior to experimentation, the crab's chelae were pinched with pliers to initiate automization and thus avoid cannibalism. The crabs were kept in a fifty-gallon fiber glass Living Stream (Frigid Units, Inc.) at a salinity of 15 ppt and at a temperature of 19°C. There was no feeding of the crabs while they were kept in the aquarium. The crabs were kept at a maximum of three days before they were used in the assay.

Reagents Used in the Assay

All reagents used were obtained from Sigma Chemical Company.

Borate Solution: A 0.8 M borate solution was prepared and adjusted to a pH of 9.0 with KOH.

Ehrlich Reagent: Ehrlich reagent was made by the addition of 0.5 gm of paradimethylaminobenzaldehyde in glacial acetic acid (50 ml) containing 1 N concentrated hydrochloric acid (43,44).

Acetylglucosamine Standard: An acetylglucosamine standard was prepared using distilled water saturated with chloroform. A 5.0 M

solution of acetylglucosamine was prepared as the standard stock solution. Dilutions of this stock solution were made and a standard curve was determined for this assay.

Procedure for Assay

The crabs were anesthetized on ice at 4°C for 15 to 20 minutes. A square portion of the dorsal epidermis, 6.25 cm^2, was cut from the cardiac region and was used as the source of tissue. The crabs were staged according to the method of Drach, modified by Passano (2). To remove the epidermis from the portion of the exoskeleton that was cut. Crabs in the premolt stage (D_1 and later) had reabsorbed the membrane layer and therefore the epidermis did not come off with the exoskeleton (42). The epidermis of these premolt crabs was pulled off the stomach mesentery and muscle.

The epidermal specimens, which averaged 10.0 mg. were floated in chilled sea water (15 ppt) until they were homogenized in 10.0 ml of sea water with a glass to glass tissue grinder in order to keep the enzymes viable. The homogenate was then centrifuged at 280 g., in a Sorval RC3 centrifuge with a HG 4 head for 10 minutes. After centrifugation, a 1.0 ml sample of the supernatant was diluted with 9.0 ml of distilled water. A 1.0 ml sample of the pellet was pipetted and resuspended in 9.0 ml of distilled water. A 1.0 ml sample of the resuspended pellet and a 1.0 ml sample of the diluted supernatant were put on ice. The remaining 9.0 ml of the resuspended pellet was recentrifugated at 1120 X g.

A 1.0 ml sample of the resulting supernatant and a 1.0 ml sample of the resulting pellet were taken. These two samples and a 1.0 ml sample of the acetylglucosamine standard were analyzed by a modified Morgan Elson method for acetylglucosamine determination (43,44). The product, formed by heating N-acetylglucosamine with an alkaline solution reacts with the p-dimethylaminobenzaldehyde (Ehrlich Reagent) and forms a distinct color. N-acetylglucosamine produces the same color but not the disaccharide N-N-diacetylchitobiose (45).

To each 1.0 ml sample in the test tubes, 0.5 ml of borate solution was added. The samples were then heated for 5 minutes in a boiling water bath. After cooling to room temperature, 5.0 ml of the Ehrlich Reagent was added to each tube. The tubes were then allowed to incubate in a water bath for 10 minutes at 37°C. The solutions in the tubes were then read on a Beckman spectrometer at 560 nm. The mean of the two samples was then recorded. These are included in Table 2.

RESULTS AND DISCUSSION

The level of endogenous N-acetylglucosamine increased in premolt stage (D_1-D_4), and fell off rapidly in postmolt (Table 2 and Figure 4). This greater level in premolt correlates with the hypothesis that chitin was being reabsorbed from the old exoskeleton and reutilized in the new cuticle. Gwinn and Stevenson demonstrated that incorporation of acetylglucosamine into chitin increased substantially in early premolt and decreased in late postmolt in crayfish (22,23). These changes paralleled the increase in UDP acetylglucosamine pyrophosphorylase, one of the enzymes in the biosynthetic pathway of chitin (22,23). Bade postulated that this enzyme arose "de novo" in the epidermal cells (46). Gwinn and Stevenson suggested that the source of acetylglucosamine was the breakdown product of old chitin. If this were true, the breakdown of the old chitin must coincide with incorporation of acetylglucosamine into chitin. Bade showed that in Manduca, the breakdown of chitin occurred relatively swiftly, and that this sharp rise in chitinase activity disappeared abruptly, approximately 12 hours later (46). N-acetylglucosamine is an obvious product which can be incorporated into the new cuticle chitin. A possible criticism of this interpretation is the possibility that glucose was a precursor of the new cuticle chitin in premolt (22,23). Several experiments along this line rendered this highly controversial. Bade and Wyatt showed that 65% of the larval chitin of the Cecropia silkworm was reabsorbed on the last day before molt (47). Approximately 25% to 30% of the new cuticle was synthesized

TABLE 2

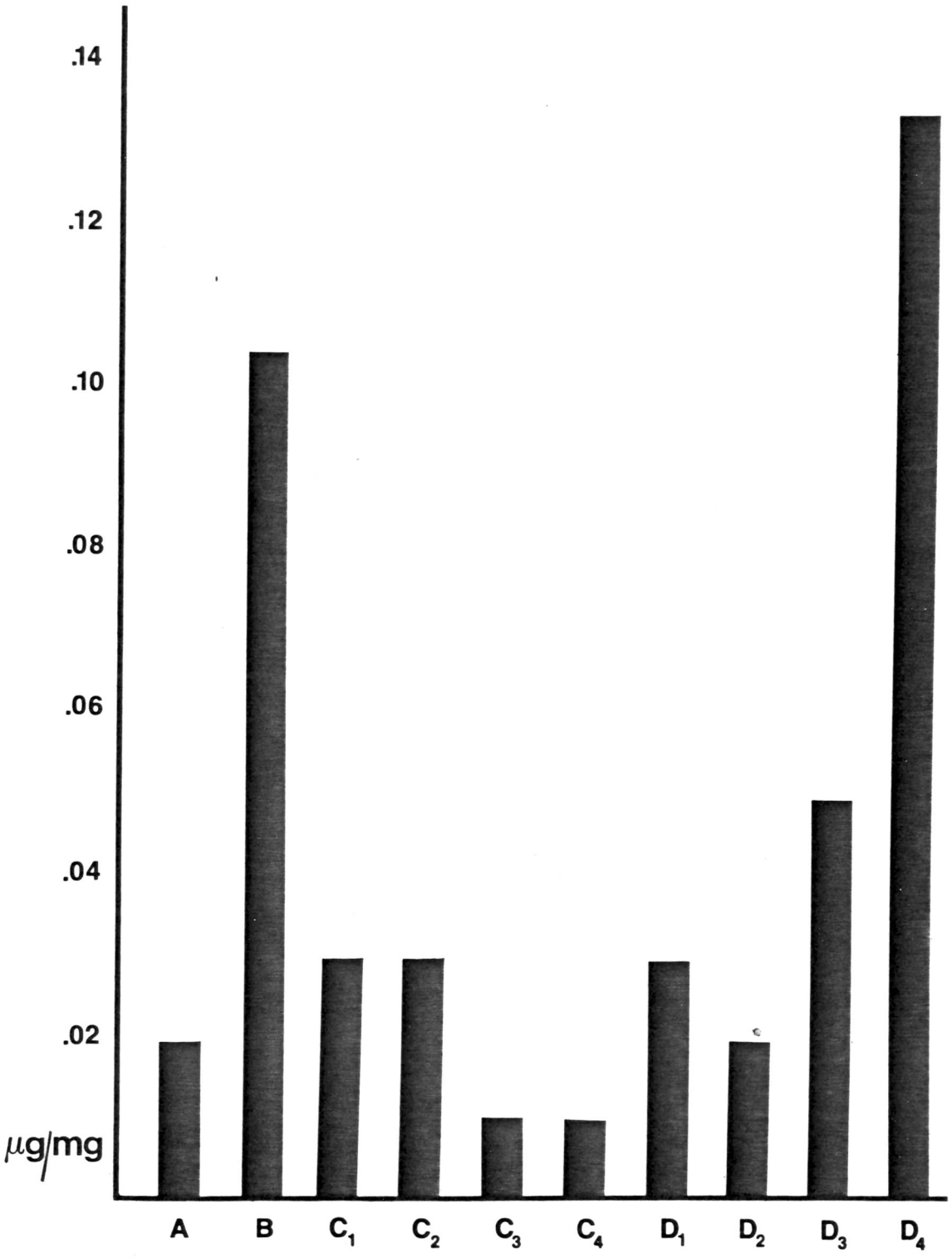
N-ACETYLGLUCOSAMINE CONCENTRATION AT VARIOUS STAGES OF MOLT CYCLE

molt stage	acetylglucosamine concentration µg/mg tissue	average concentration	standard deviation
A	.00, .03, .01	.02	.02
B	.19, .00	.10	.13
C_1	.02, .05, .01, .01	.03	.02
C_2	.02, .06, .10	.03	.03
C_3	.01, .01, .01, .00, .01, .01, .00, .01, .01, .01, .01, .00, .01, .00, .01	.01	.01
C_4	.01, .01, .01, .00, .01, .00, .01, .01, .01, .01, .00, .01, .00, .01	.01	.00
D_1	.01, .00, .02, .02, .02, .03, .05, .04, .04, .03	.03	.01
D_2	.01, .00, .02, .01, .05, .11, .13, .14, .10, .17, .14, .11, .06, .04, .25, .34, .40, .18, .10, .80, .70, .13, .21, .05	.02	.20
D_3	.03, .04, .06, .01, .07, .06, .09, .02, .02	.05	.03
D_4	.33, .17, .09, .16, .32, .20, .03, .19, .18, .01, .02, .08, .04, .00	.13	.11

FIGURE 4

ACETYLGLUCOSAMINE LEVELS DURING VARIOUS

STAGES OF MOLT CYCLE


.14
.12
.10
.08
.06
.04
.02
μg/mg
A
B
C1
C2
C3
C4
D1
D2
D3
D4

during premolt in crayfish (22,23). Several workers showed that very little glucose was incorporated prior to ecydsis, not enough to account for the amount of chitin formed during this period (47,48). It seems more likely that glucose was not a major precursor for new cuticle chitin in the premolt organism. Gwinn and Stevenson postulated that N-acetylglucosamine was the major precursor of chitin in premolt (22,23). The activity of the biosynthetic enzymes increased in premolt (22,23).

The results of the experiment presented here demonstrated a change in the content of endogenous N-acetylglucosamine depending on the molting stages. It is certainly likely that the levels of acetylglucosamine were a reflection of the level of chitinase activity within the epidermal cells. The assay in this study obviously did not distinguish between chitinase and chitobiase. The results of this experiment specifically showed a slight drop in endogenous N-acetylglucosamine at the D_3 molt stage. A drop in chitinase activity was noted by others in the same stage (51,46). Gwinn and Stevenson interpreted that this drop was due to apolysis that occurred at this stage (22,23). A study is needed to determine whether change in levels of chitinase indeed takes place in molting of the blue crab.

SUMMARY

The results of this study support the hypothesis that the levels of N-acetylglucosamine varies with the molting stages of the blue crab Callinectes sapidus Rathbun. The increase in N-acetylglucosamine in premolt correlated with the partial digestion of the old cuticle chitin. The lowering of N-acetylglucosamine levels in postmolt coincided with the formation of new cuticle during which N-acetylglucosamine could be utilized to form new cuticle chitin.

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