

Changing Activities of the Crustacean Epidermis during the Molting Cycle

J. ROSS STEVENSON

Department of Biological Sciences, Kent State University, Kent, Ohio 44242

SYNOPSIS. The criteria established by Drach for subdividing the molting cycle into stages are reviewed, and a suggestion is made for improving the uniformity of postmolt staging of different species. Changes in the epidermis during the molting cycle of the crayfish *Orconectes obscurus* and *O. sanborni* are described. Epidermal DNA content was measured throughout the cycle and found to drop sharply at stage D₀ and to rise sharply at stage A. Protein content declined during postmolt and rose during premolt, as expected. Protein synthesis remained more or less constant during postmolt, rose during premolt, and dropped to the postmolt level at ecdysis. Chitin synthesis appeared to follow two different curves depending upon whether labelled glucose or acetylglucosamine was used as precursor. This, and the presence of a separate enzyme capable of phosphorylating acetylglucosamine and not glucose, suggests that acetylglucosamine may be utilized directly without prior conversion to glucose. Actinomycin D was found to prevent increases in rate of chitin biosynthesis during premolt but not to inhibit chitin biosynthesis already underway. During the same period, actinomycin stimulated general protein biosynthesis. By utilizing the molt staging criteria described, we were able to detect induction of premolt by ecdysone.

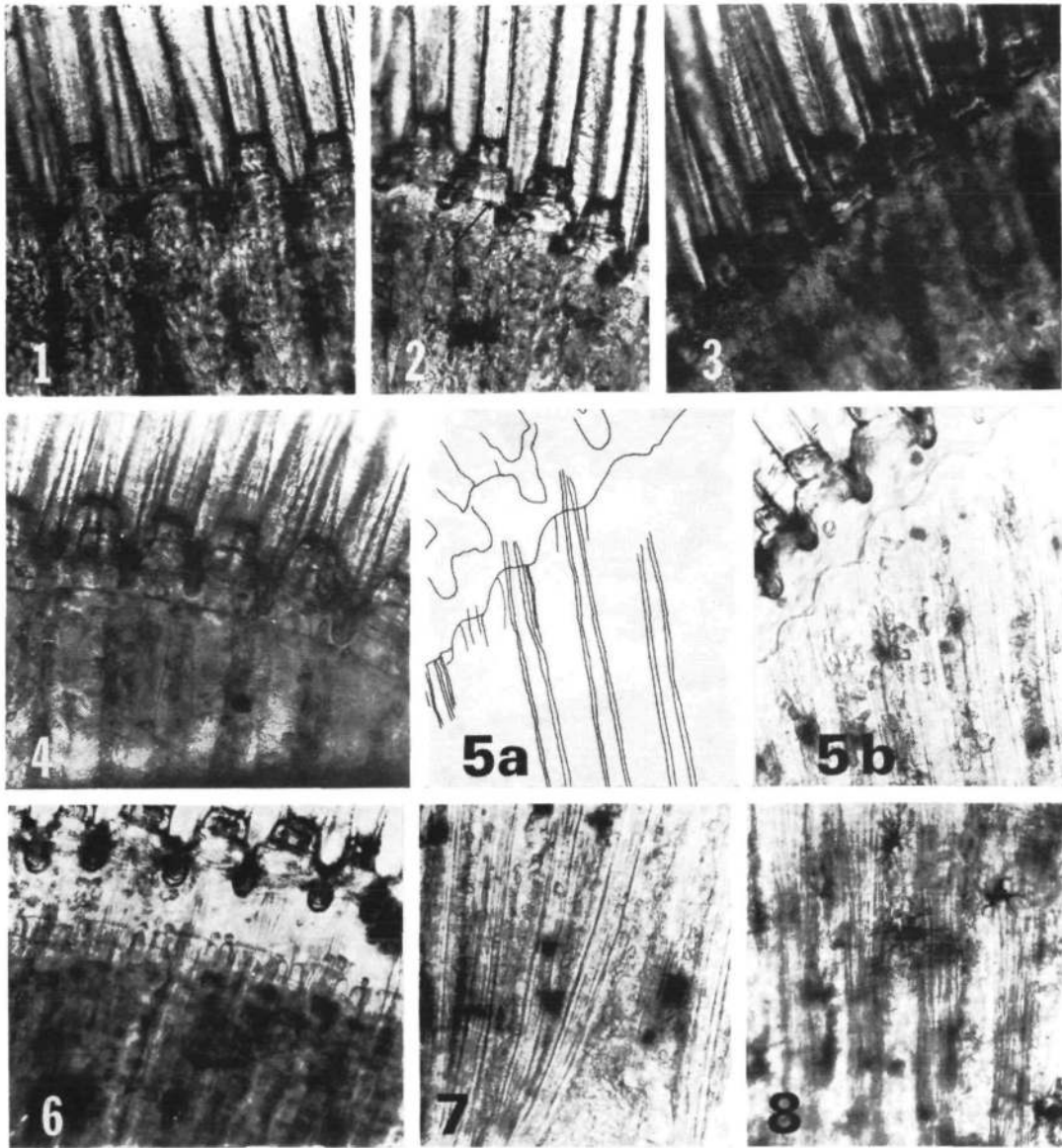
We have been studying the biosynthesis of nucleic acids, protein, and chitin in the crayfish epidermis in relation to the molting cycle and the effects of ecdysone. Before describing this work, I should like to review briefly the criteria established by Drach (1939, 1944) for subdividing the molting cycle into stages, because the precise determination of stages is essential for this kind of study. I also wish to make some recommendations concerning the postmolt staging.

Concerning the postmolt and intermolt stages first, four of the criteria of Drach are universal; that is, they can be directly applied to any crustacean (see Table 1). These are the criteria for stages A₁, C₃, C₄', and C₄. The rest of the postmolt stages are identified by increasing degrees of rigidity of the different parts of the body, except in poorly calcified species, in which stage C is not subdivided (Drach and Tchernigovtzeff, 1967). Since different parts of different crustaceans become rigid at different times after the molt, there is

no uniformity from species to species in the meaning of the different stages based on rigidity. In the interest of greater uniformity, I propose adding two more universal criteria, the criteria for determining stages A₂ and C₁. These criteria, shown on Table 1, can be recognized when stained sections of the cuticle are made. Once it is known when these changes take place, then their corresponding external changes can be used in the routine staging of the animal. This is much more meaningful than arbitrarily selecting different criteria for each species. The determination of the other postmolt stages (B₁, B₂, and C₂) is still arbitrary (Stevenson, 1968).

Premolt stage criteria are shown on Table 2. The earliest sign that premolt has begun is apolysis, the detachment of the epidermis from the cuticle. Next, new setae form underneath the old, and soon after they are completed, secretion of new cuticle begins (Drach, 1939). To detect apolysis and formation of the new setae in the living animal, one must examine some part of the body where the cuticle is sufficiently transparent. In brachyuran crabs, setae can be seen by cutting a hole in the brachiolegite and removing and examin-

The work reported in this paper was supported in part by research grant GB-8688 from the National Science Foundation.



FIGURES 1-8. Bases of crayfish setae at the edge of an uropod or the telson.

FIG. 1. Stage C_4 .

FIG. 2. Early stage D_0 . Arrow indicates the edge of the epidermis.

FIG. 3. Stage D_0 . Arrow indicates edge of the epidermis.

FIG. 4. Stage D_1' .

FIG. 5. Stage D_1'' . Tubular nature of the developing setae is visible. 5a is a tracing of part of

5b, showing the principal features.

FIG. 6. Stage D_1''' , showing tips of completed new setae.

FIG. 7. Stage D_1''' , showing hairs on the shafts of the new setae.

FIG. 8. Stage D_2 . The shafts of the new setae appear more bushy due to the appearance of more hairs.

ing a piece of an epipodite, a cumbersome procedure that cannot be repeated often. In the crayfish, they can be seen without

injury to the animal by holding the entire animal on the stage of a microscope and examining the edge of a uropod or the

TABLE 1. *Postmolt stages and intermolt.*

Stage	Begins when
A1	Animal molts.
A2	Material is injected into pre-exuvial layers.
B1
B2
C1	Chemical changes in pre-exuvial layers are complete.
C2
C3	Integument has achieved its full rigidity.
C4'	Secretion of principal layer is complete and secretion of membranous layer begins.
C4	Secretion of membranous layer is complete.

telson. Figure 1 shows the bases of the setae before apolysis begins. In Figure 2, apolysis has begun; the arrow indicates the edge of the epidermis. In Figure 3, the epidermis has withdrawn further, but it still touches the bases of the old setae. Both of these are in stage D₀ (Drach and Tchernigovtzeff, 1967). Stage D₀ may also be recognized by measuring regeneration of limb buds over a period of time. Resumption of regeneration after a period without growth (growth plateau) indicates the beginning of premolt (stage D₀) (Skinner, 1962; Stevenson and Henry, 1971).

Stage D₁, the next stage of premolt, can be subdivided according to stages in the formation of the new setae. Subdivision of this stage is especially useful because this is the important period when the epidermis is constructing the machinery for secretion of new cuticle. The three subdivisions, stages D₁', D₁'', and D₁'', as we described them (Stevenson et al., 1968) on the basis of Drach (1944), are illustrated in Figures 4, 5, and 6, respectively. Unfortunately, at about the same time we described these substages in the crayfish,

TABLE 2. *Premolt stages.*

Stage	Begins when
D0	Apolysis occurs, regenerating limb buds begin rapid growth, and gastroliths begin to be formed.
D1	Formation of new setae begins.
D2	Secretion of new cuticle begins.
D3	Much of old post-exuvial endocuticle has been reabsorbed.
D4	The line of dehiscence opens.

Drach and Tchernigovtzeff (1967) published a revision of these substages, and our description does not agree with their revision exactly.

Stage D₂ begins when secretion of the new cuticle begins. In the crayfish, no change in the region between the new setae can be seen at this time, but the new setae themselves change in appearance. They appear bushier (compare Figs. 7 and 8).

Now let us turn to a consideration of the changing biosynthetic activities of the epidermis during the molting cycle of the crayfish *Orconectes obscurus* and *O. sanborni*. First, we measured the DNA content of the epidermis in order to be able to express other changes on a per cell basis (Humphreys and Stevenson, unpublished). DNA content per mg wet weight of epidermis is shown in Figure 9. There is a dramatic drop in DNA concentration at stage D₀ and a rise at stage A. The drop at D₀ may reflect the cell enlargement which occurs at this time (Travis, 1955;

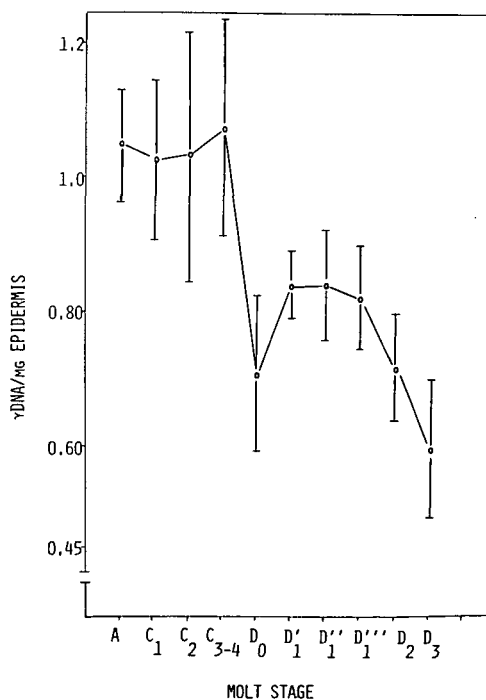


FIG. 9. Epidermal DNA content during the molting cycle.

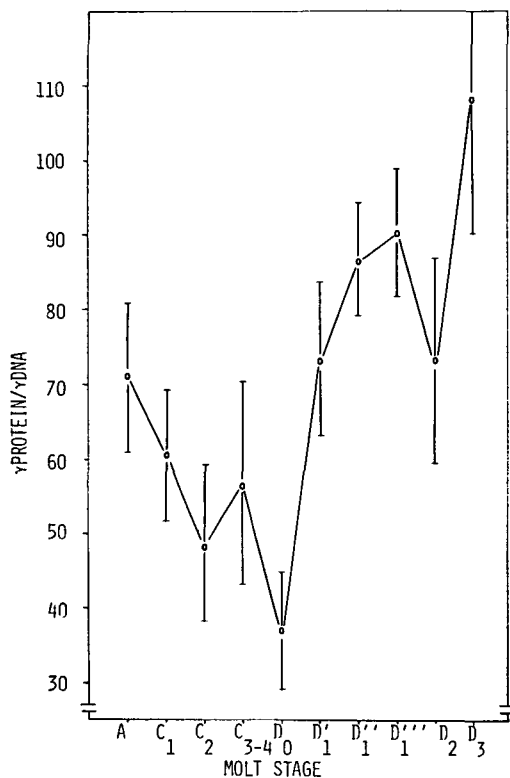


FIG. 10. Epidermal protein content during the molting cycle.

Dennell, 1960; Skinner, 1962; Stevenson et al., 1968). It has been reported that mitosis occurs during stages D₀, D₁', and D₁'' (Tchernigovtzeff, 1959), but it is not yet known when DNA synthesis occurs. The sharp rise in the curve at stage A may be due to DNA synthesis at this time, perhaps in response to ecdysone. A high ecdysone titer at stage A has been reported by Adelson (1969) and by Faux et al. (1969), and stimulation of DNA synthesis by ecdysone has frequently been reported in insects.

Protein content of the epidermis per unit weight DNA was also measured, and is shown in Figure 10. Protein content of the cells declined during postmolt, as expected. During postmolt, the cells were decreasing in size and their rate of cuticle secretion was decreasing. Protein content was lowest at stage D₀ when the cells were enlarging. This suggests that initial cell enlargement is not due to synthesis of new

protoplasm but perhaps to the absorption of water. Protein content increased during premolt as the cells synthesized new protoplasm, new enzymes, and, beginning at stage D₂, cuticle protein.

Figure 11 represents protein synthesis, expressed as decays per minute of ¹⁴C-leucine incorporated into protein in one hour per unit weight DNA (Humphreys and Stevenson, unpublished). Epidermal protein synthesis has also been studied by Skinner (1965) and by McWhinnie and Mohrherr (1970), but not at every stage of the molting cycle. We found a more or less constant rate of synthesis during postmolt even though protein content was decreasing (Fig. 10). Therefore, specific activity was increasing, as shown in Figure 12. At the same time, the loss of protein to the new cuticle by secretion was decreasing. Therefore, catabolism of protein must have been increasing correspondingly. Protein synthesis increased during premolt, as expected (Fig. 11). Changes in specific activity, shown on Figure 12, agree both with Skinner (1965) and with McWhinnie and Mohrherr (1970). Skinner reported a rise in incorporation from stage C₄ to D₀₋₁₋₂ and McWhinnie and

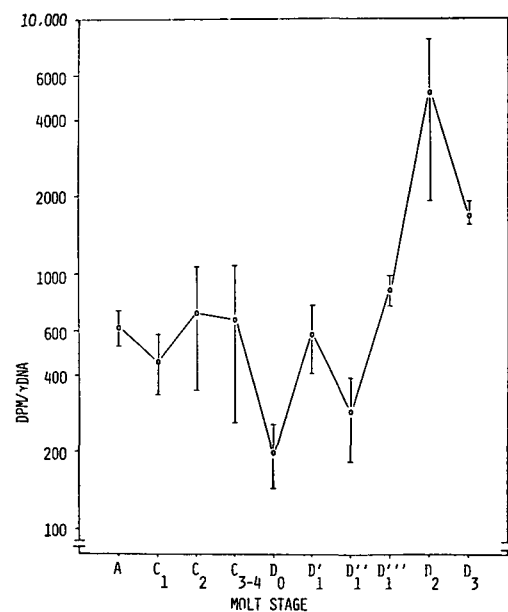


FIG. 11. Epidermal protein synthesis during the molting cycle.

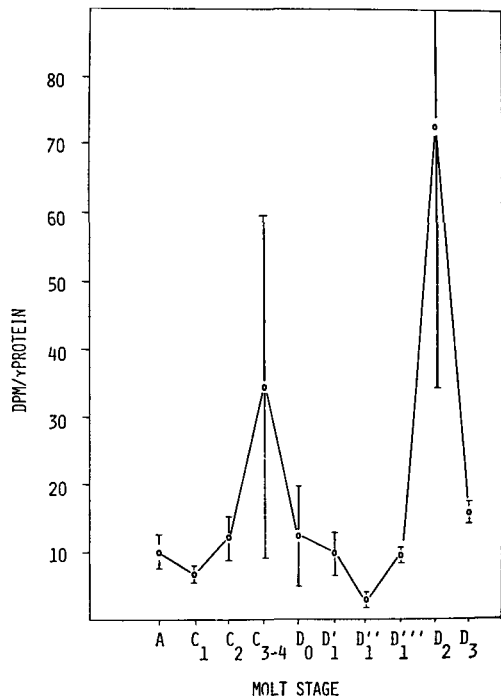


FIG. 12. Specific activity of epidermal protein during the molting cycle.

Mohrherr reported a drop from C₃₋₄ to D₀₋₁.

We also studied the changes in rate of chitin biosynthesis during the molting cycle (Hornung and Stevenson, 1971). We injected ¹⁴C-glucose and 12 hours later isolated chitin from the whole animal. Figure 13 shows the results, plotted on a logarithmic scale. The rate of chitin biosynthesis was always higher than background, even at stage C₄, when it has been supposed that cuticle secretion stops. Neville (1965) found that three species of insects that he examined form their cuticles in daily growth layers, and he suggested that the phenomenon may be general in arthropods. Our unpublished observations seem to confirm this idea for the crayfish. A new lamina seems to be formed each day. At stage C₄, the innermost laminae are extremely thin (Stevenson et al., 1968). Perhaps these very thin laminae continue to be secreted, one each day throughout stage C₄. New chitin may also be added to laminae already secreted. If ¹⁴C-glucose is injected at any time during postmolt, the

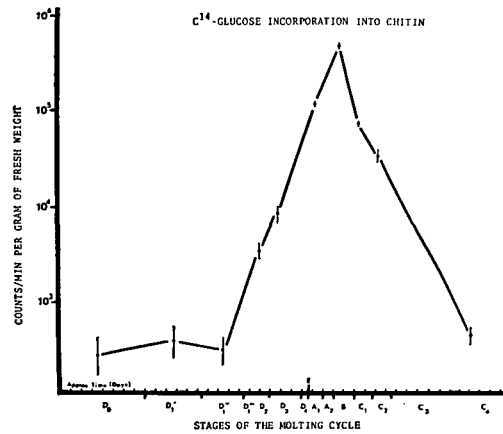


FIG. 13. Incorporation of ¹⁴C-glucose into chitin. Ordinates are plotted on a logarithmic scale.

chitin in layers formed prior to the previous molt becomes labelled as well as chitin in the new layers (Gwinn and Stevenson, unpublished).

It will be noted in Figure 13 that chitin synthesis continued even during early premolt, when the epidermis separates from the cuticle. The explanation may be that the epidermis does not separate from the cuticle of all parts of the body at once. The rate of synthesis increased during later premolt, when secretion of new cuticle began, and reached a peak at postmolt stage B, as expected from the histological observation that the cuticle seems to thicken fastest at stage B (Stevenson, unpublished). One result which does not seem to

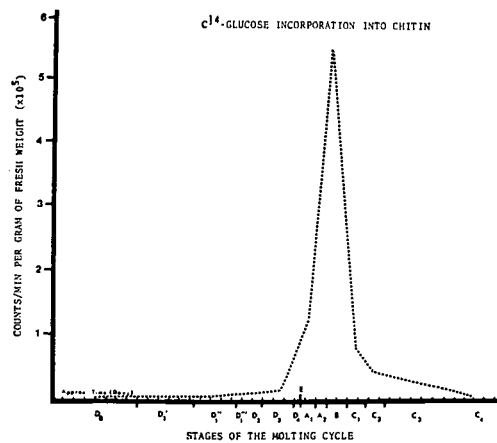


FIG. 14. Incorporation of ¹⁴C-glucose into chitin plotted on a linear scale.

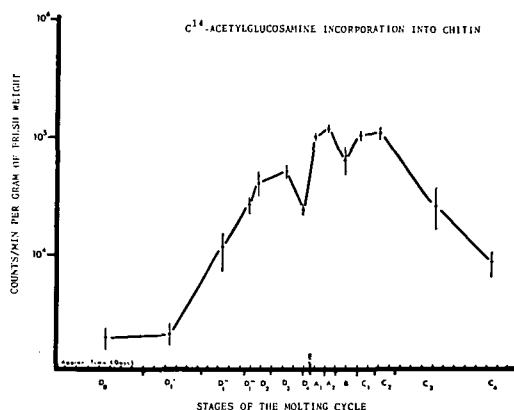


FIG. 15. Incorporation of ^{14}C -acetylglucosamine into chitin. Ordinates are plotted on a logarithmic scale.

confirm histological observations is the low rate of incorporation of label during late premolt. This is seen more clearly when the data are plotted on a linear scale (Fig. 14). Could it be that the animal uses acetylglucosamine (AGm) derived from breakdown of the old cuticle in preference to glucose during premolt? We repeated the incorporation experiment using ^{14}C -AGm instead of glucose (Gwinn and Stevenson, unpublished); the results (Fig. 15) seem to confirm the hypothesis. AGm was utilized at a high rate significantly earlier in premolt than was glucose. It was also used with greater efficiency than glucose during both premolt and postmolt. The different shapes of the incorporation curves suggest different incorporation pathways. Figure 16 shows in abbreviated form the pathway by which glucose is converted to chitin. If AGm is deacetylated

and deaminated to glucose, then its incorporation would follow the same pathway as glucose. If, on the other hand, it is phosphorylated directly, it could enter the pathway at a later point. To find whether this is possible, we tested crude epidermal homogenate for AGm kinase activity. We incubated ^{14}C -AGm with homogenate in a suitable buffer containing ATP, then looked for and found labelled AGm-6-phosphate on thin layer chromatograms (Gwinn and Stevenson, unpublished). Glucose did not inhibit the reaction. Therefore, the kinase appears to be a specific enzyme, the substrate for which is AGm and not glucose. This lends support for the hypothesis that AGm is utilized directly without conversion first to glucose.

As a beginning in attempting to understand how chitin biosynthesis is controlled in the crayfish, we investigated the effect of actinomycin D on this process. Since actinomycin inhibits RNA synthesis, any inhibition of chitin synthesis would be indirect evidence that chitin synthesis depends on RNA synthesis. To study this, we injected actinomycin D and ^{14}C -glucose into crayfish at the same time and extracted and counted the chitin after 24 hours. The results are shown on Fig. 17 (Stevenson and Tung, 1971). Significant inhibition was obtained at stages D_1''' and D_2 and not at stages D_1' and A. These results suggest that new RNA is not needed for the low level of chitin synthesis which occurs at stage D_1' and also not for the high level of synthesis at stage A, when the rate of synthesis has nearly reached its

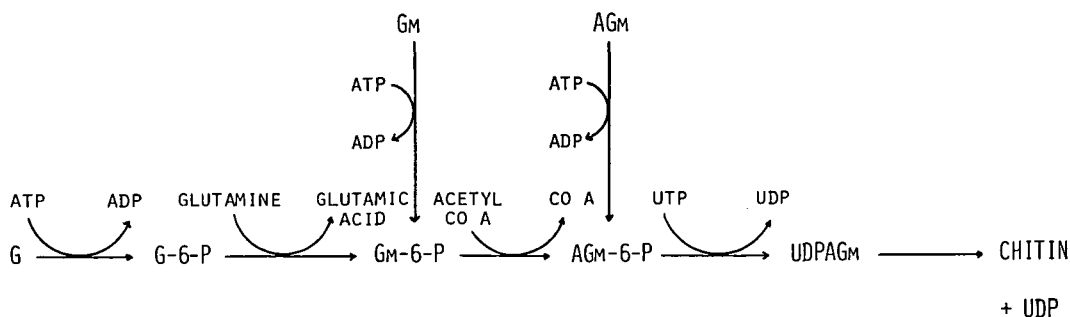


FIG. 16. The pathway of chitin biosynthesis, showing the postulated point of entrance of ac-

tylglucosamine. G = glucose, Gm = glucosamine, AGM = acetylglucosamine, -6-P = 6-phosphate.

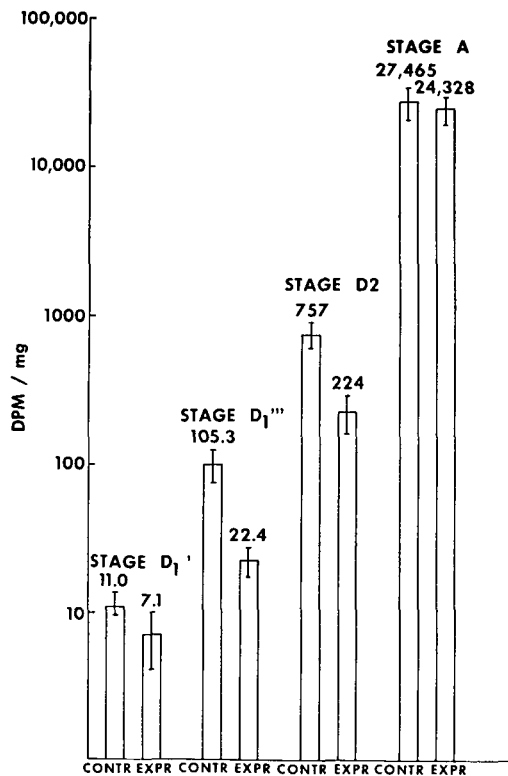


FIG. 17. Effect of actinomycin D on chitin biosynthesis at different molting stages, plotted on a logarithmic scale. Contr = controls, Expr = experimentals = actinomycin-injected.

peak. It seems that RNA synthesis may be needed only when the rate of chitin biosynthesis is increasing. If so, then the RNA species involved or the chitin synthesizing enzymes synthesized from these RNA species or both must be very stable because chitin synthesis continues for two weeks or more after stage A. If no more RNA for chitin synthesis is synthesized after stage A, then the declining rate of chitin biosynthesis during postmolt may be due to the gradual breakdown of these RNA's or enzymes.

The data also show that actinomycin never reduced the rate of chitin biosynthesis to a level below that of the controls in the preceding molting stage studied. In other words, actinomycin prevented an increase in the rate of synthesis during the period of actinomycin treatment, but it apparently did not reduce it below the level

it had already achieved. This result supports the notion that the RNA or chitin synthesizing enzymes or both are very stable because no significant breakdown occurred during the treatment period.

After finding that actinomycin D inhibited the increase in chitin biosynthesis which occurs during premolt, we confidently expected it to inhibit the increase in total protein biosynthesis during premolt also. However, we obtained the opposite result when we injected actinomycin D into crayfish 5 to 22 hours prior to injecting ¹⁴C-leucine and isolating and counting epidermal protein 2 to 5 hours later (Stevenson and Tardif, unpublished). In these several experiments, actinomycin D stimulated protein biosynthesis fourfold during stage D₀ and up to twofold during stage D₁'. In stage D₂, on the other hand, it inhibited protein biosynthesis by about 30%. Perhaps these results reflect the influence of a mechanism that controls protein biosynthesis at the translation level. There may be, for instance, an RNA synthesis-dependent unstable translation repressor such as that postulated by Garren et al. (1964) and others. If so, the antibiotic would stimulate protein synthesis by inhibiting synthesis of the repressor, which is more unstable than the RNA species needed for general protein synthesis. Such a mechanism could control the increase in protein biosynthesis observed to occur during premolt (Fig. 11). If progressively less of the inhibitor were synthesized during

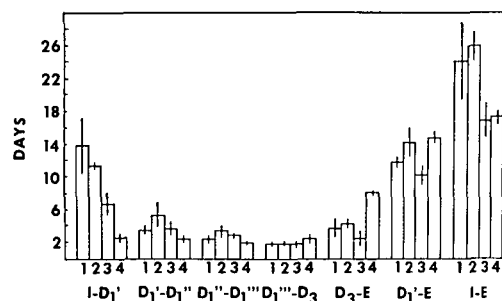


FIG. 18. Responses of form II male crayfish to α -cydysone in the summer. Group 1, control; groups 2, 3, and 4 received 0.0214, 0.214, and 2.14 μ g edcysone/g body weight, respectively, in a single injection.

pre-molt, protein synthesis would increase. The fact that actinomycin produced less stimulation and, finally, inhibition as pre-molt proceeded supports this hypothesis.

The criteria for molt staging described at the beginning of this review have been useful in assaying ecdysone activity as well as in studying changing rates of biosynthesis, and we were able to follow the effect of injected ecdysone on the progression of the molting stages by using these criteria (Warner et al., 1969; Warner and Stevenson, 1972). Figure 18 shows the effects of injecting three different doses of α -ecdysone into intermolt crayfish in the summer. The control animals molted as well as the animals injected with the hormone, and the animals injected with 2.14 or 0.214 μ g ecdysone per gram body weight molted sooner than the controls. The effect of the hormone showed up much earlier than molting, however. It showed up in the length of time taken the animals to enter pre-molt. As shown by the figure, the animals injected with the hormone entered stage D_1' sooner than the controls, and the time taken to enter this stage varied inversely with the hormone dose. This result compares with that of Krishnakumaran and Schneiderman (1970), who detected apolysis in the crayfish *Procambarus* by cutting sections of integument at intervals after ecdysone injection.

REFERENCES

- Adelung, D. 1969. Die Ausschüttung und Funktion von Häutungshormon während eines Zwischenhäutungs-Intervalls bei der Strandkrabbe *Carcinus maenas* L. Z. Naturforsch. 24:1447-1455.
- Dennell, R. 1960. Integument and exoskeleton, p. 449-472. In T. H. Waterman [ed.], The physiology of crustacea. Vol. I. Academic Press, New York.
- Drach, P. 1939. Mue et cycle d'intermue chez les crustacés décapodes. Ann. Inst. Océanogr. (Monaco) 19:103-391.
- Drach, P. 1944. Etude préliminaire sur le cycle d'intermue et son conditionnement hormonal chez *Leander serratus* (Pennant). Bull. Biol. Fr. Belg. 78:40-62.
- Drach, P., and C. Tchernigovtzeff. 1967. Sur la méthode de détermination des stades d'intermue et son application générale aux crustacés. Vie Milieu Ser. A: Biol. Mar. 18:595-610.
- Faux, A., D. H. S. Horn, E. Middleton, H. M. Fales, and M. E. Lowe. 1969. Moulting hormones of a crab during ecdysis. Chem. Commun. (London) p. 175-176.
- Garren, L. D., R. R. Howell, G. M. Tomkins, and R. M. Crocco. 1964. A paradoxical effect of actinomycin D: the mechanism of regulation of enzyme synthesis by hydrocortisone. Proc. Nat. Acad. Sci. U.S. 52:1121-1129.
- Hornung, D. E., and J. R. Stevenson. 1971. Changes in the rate of chitin synthesis during the crayfish molting cycle. Comp. Biochem. Physiol. 40:341-346.
- Krishnakumaran, A., and H. A. Schneiderman. 1970. Control of molting in mandibulate and chelicerate arthropods by ecdysones. Biol. Bull. 139:520-538.
- McWhinnie, M. A., and C. J. Mohrherr. 1970. Influence of eyestalk factors, intermolt cycle and season upon 14 C-leucine incorporation into protein in the crayfish (*Orconectes virilis*). Comp. Biochem. Physiol. 34:415-437.
- Neville, A. C. 1965. Circadian organization of chitin in some insect skeletons. Quart. J. Microsc. Sci. 106:315-325.
- Skinner, D. M. 1962. The structure and metabolism of a crustacean integumentary tissue during a molt cycle. Biol. Bull. 123:635-647.
- Skinner, D. M. 1965. Amino acid incorporation into protein during the molt cycle of the land crab, *Gecarcinus lateralis*. J. Exp. Zool. 160:225-234.
- Stevenson, J. R. 1968. Metecdysial molt staging and changes in the cuticle in the crayfish *Orconectes sanborni* (Faxon). Crustaceana 14:169-177.
- Stevenson, J. R., R. H. Guckert, and J. D. Cohen. 1968. Lack of correlation of some proecdysial growth and developmental processes in the crayfish. Biol. Bull. 134:160-175.
- Stevenson, J. R., and B. A. Henry. 1971. Correlation between the molt cycle and limb regeneration in the crayfish *Orconectes obscurus* (Hagen) (Decapoda Astacidea). Crustaceana 20:301-307.
- Stevenson, J. R., and D. A. Tung. 1971. Inhibition by actinomycin D of the initiation of chitin biosynthesis in the crayfish. Comp. Biochem. Physiol. 39B:559-567.
- Tchernigovtzeff, C. 1959. Croissance épithéliale et cycle d'intermue chez *Leander serratus* (Pennant). C. R. Acad. Sci. 248:600-602.
- Travis, D. F. 1955. The molting cycle of the spiny lobster, *Panulirus argus* Latreille. II. Pre-ecdysial histological and histochemical changes in the hepato-pancreas and integumental tissues. Biol. Bull. 108:88-112.
- Warner, A. C., J. R. Stevenson, and C. Church. 1969. The initiation of precocious pre-molt in *Orconectes obscurus* with injections of α -ecdysone and crustecdysone. Amer. Zool. 9:1085.
- Warner, A. C., and J. R. Stevenson. 1972. The influence of ecdysones and eyestalk removal on the molt cycle of the crayfish, *Orconectes obscurus*. Gen. Comp. Endocrinol. (In press)