Macromolecular Changes Associated with the Growth of Crustacean Tissues

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SYNOPSIS. Tissue mass, rate of protein synthesis, content of ribosomal RNA and rates of synthesis of ribosomal RNA have been studied throughout the molting cycle in the midgut gland, epithelium, and somatic muscle in the land crab, Gecarcinus lateralis. In all tissues there is an increase in ribosomal RNA followed by an increase in the rate of synthesis of protein in the premolt period. Subsequently, the three tissues differed in that (a) in the midgut gland the level of ribosomal RNA and protein synthesis returned to the intermolt rates before ecdysis whether or not the mass of the tissue was increasing or decreasing; (b) ribosomal RNA and protein synthesis in epithelium reached a maximum at a time when epithelial cells reached a maximal size; subsequently, all three parameters decreased toward intermolt levels before ecdysis; (c) ribosomal RNA and protein synthesis reached a maximum in the premolt period in somatic muscle while the muscle was in fact decreasing in mass. Muscle ribosomes are very stable and appear to be conserved for weeks or months to be reused after ecdysis in a second burst of protein synthetic activity at the time when there is replacement and growth of new muscular tissue. The relation of these events with hormonal control of growth is discussed.

For our studies on growth and its control, we have selected the land crab, Gecarcinus lateralis, as our experimental animal since, as in other arthropods, its growth is restricted to the molting period. Figure 1 is an idealized sketch of the changes in total body mass as the animal progresses from one intermolt to the next. There is a gain in weight during the premolt period (Skinner, 1962) due largely to the uptake of water stored in pericardial sacs (Bliss and Boyer, 1964). On the day of the ecdysis the animal loses weight as it sheds its old exoskeleton. Gecarcinus is conservative, however, and a day or so after ecdysis, it eats its old exoskeleton. In the next intermolt stage, the animal's weight is greater than its previous intermolt weight, but somewhat less than the maximal weight reached in the premolt period. As will be described below, the net uptake of water and the net synthesis of protein do not necessarily occur at the same time; therefore, this pattern of changes of weight during the molt cycle does not give direct information on when growth is occurring.

Molting is controlled by a hormone secreted by the Y-organ (Echalier, 1954, 1955). A large body of evidence has accumulated that molting in insects is controlled by the hormone, ecdysone. Since ecdysone has been isolated from certain crustaceans (Karlson and Skinner, 1960) it is assumed by analogy to be the molting hormone in crustaceans as well. During the intermolt periods, the action of the molting hormone is inhibited by the molt-inhibitory hormone, a neurosecretion of the X-organ. The interplay between the two hormones is not understood; possibly the secretions of the X-organ destroy ecdysone. In nature, molting is probably initiated by the cessation of the secretion of the molt-inhibitory
hormone by the X-organ. Since the neurosecretory cells of the X-organ are located in the eyestalks, their inhibition can be removed by extirpating the eyestalks. This simple surgical procedure initiates preparations for ecdysis and the concomitant growth of the animal.

In the two-month premolt period characteristic of large specimens (80 to 110 g) of Gecarcinus, the preparations for ecdysis pass through a number of "points of no return." For example, if a crab autotomizes a walking leg either before removal of the eyestalks or within a certain critical period after their removal, it forms a limb bud. By the time ecdysis occurs, the regenerated limb has developed into a new walking leg. Conversely, if a crab autotomizes a limb three or more weeks after removal of the eyestalks, no limb bud appears during the last six or more weeks of that premolt period. Obviously, the factors which regulated the formation of a limb bud are changing during the premolt period.

In order to study the problem of growth and its hormonal control, we thought it necessary first to determine when growth actually occurs in various tissues, and then to examine some of the macromolecular changes associated with growth. These experiments are described here.

The difficulties in defining growth are well known. For purposes of this discussion, we will equate growth with net synthesis of protein. We have studied protein synthesis in vivo and the associated changes in ribosomal RNA. Therefore, I will review some of the functions of ribosomal RNA in protein synthesis. At the top of Figure 2 is a diagram of a polysome, the unit on which protein is synthesized. The polysome is composed of ribosomes attached to a strand of messenger RNA. The messenger RNA contains in its base sequence the information for the amino acid sequence of the protein being synthesized. Also attached to the polysomes are molecules of transfer RNA carrying the appropriate amino acid. These are not shown in the figure. Ribosomes released from the polysome are duplex structures characterized by a sedimentation coefficient of approximately 80 Svedberg units. The 80 S ribosome can be dissociated into its two components of 60 and 40 S. The 60 S ribosome contains RNA of about 28 S, while the 40 S ribosome contains RNA of 16 or 18 S. Ribosomal RNA makes up approximately 85 per cent of the total RNA found in the cell.

Centrifugation of isolated RNA (method of isolation modified from Hiatt, 1962) through a sucrose density gradient separates the 28 S and 18 S components of ribosomal RNA from each other and from the 4 S transfer RNA. Since the 60 S and 40 S ribosomes occur in equal numbers there is 1.5 times as much RNA in a 60 S ribosome as in a 40 S ribosome; therefore, we look for a 3:2 ratio in the total optical density units in the 28:18 S peaks obtained from the sucrose density gradients. This ratio is the criterion of a good preparation in which there has been no significant breakdown of RNA during its isolation. We have not yet attempted to isolate messenger RNA, which is present in very small quantities in most cells.

In Gecarcinus we have examined three tissues (midgut gland, epithelium, and somatic muscle) at various times during the...
GROWTH IN *Gecarcinus*

molt cycle. For each of these tissues, we have asked the questions: Is there net gain or loss of tissue in time? Can this gain or loss be correlated with changes in the rate of protein synthesis? Can changes in the rate of protein synthesis be correlated with changes in the amount of tissue RNA and in the rates of RNA synthesis? Of particular interest in obtaining information about the endocrine control of molting: do all of these changes show the same pattern in time in all three tissues?

After discussing the three tissues separately, I shall attempt to draw some general conclusions about patterns of growth in this animal.

**MIDGUT GLAND**

Among animals of a given size, there is great variability in the size of the midgut gland, determined in large part by the quantity of food eaten. Since well-fed animals clearly survive removal of the eyestalks far better than the less well-fed (Smith, 1940), we feed both experimental animals and their controls daily. Under this regimen, the midgut glands of intermolt animals fresh from the field can double in weight in a few months in the laboratory. In the late premolt period, but several weeks before ecdysis, *Gecarcinus* stops eating altogether. Although it is likely that the midgut gland serves as a food reservoir at this time, our well-fed animals do not show very remarkable decreases in the size of the midgut gland during the aphagic period. The feeding required to prevent death of eyestalkless animals leads to changes in size and total protein of the midgut gland which may well obscure the changes associated with normal molting. Hence, it is difficult to delineate the growth period for this organ.

However, the rate at which amino acids are incorporated into protein in the midgut gland is clearly related to the onset of molting (Fig. 3). The intermolt animal incorporates at a low rate with only small deviations from this rate regardless of whether the organ is enlarged or not. Since the intermolt midgut gland can double in

**EPITHELIUM**

In epithelium, the second tissue we have

examined, the maximum growth period is much more readily defined. At the beginning of the second half of the premolt period (stage D₃) the epithelial cells of *Gecarcinus lateralis* increase in volume, reaching some 25-fold by stage D₂, the stage in which they synthesize the epi- and exocuticles. In stage D₄, immediately before ecdysis, the cells shrink again. After a further decrease in size, the cells carry out their postmolt synthesis of endocuticle, but at a much slower rate than their premolt laying down of epi- and exocuticles. Although much of the protein synthesized by premolt epithelium is extruded into the cuticle as a secretory product, the epithelial cells themselves multiply, since animals differing more than five-fold in size all have epithelial cells of the same dimensions. The postmolt epithelium encompasses a larger animal than the premolt epithelium; thus, these cells have apparently grown by division during the late premolt period (Skinner, 1962).

Knowing these facts, then, we were not surprised to find that the incorporation of radioactive amino acids into epithelial protein reached a maximum rate in the late premolt period, at the time of synthesis of the new protein-rich exocuticle (Fig. 4). Again, this high point of protein synthesis is associated with the formation of new ribosomal RNA, but in this case new RNA synthesis precedes protein synthesis by some weeks. By stage D₃ in the premolt period, the RNA content of epithelium is three to four times the intermolt levels (Skinner, 1966). These findings give quantitative support to our histological finding that during the premolt period, the epithelial cells show a marked increase in basophilia and can be removed by ribonuclease. After completion of the epi- and exocuticles, when the epithelial cells shrink just prior to ecdysis, there is a loss of ribosomal RNA. Apparently, fewer ribosomes are needed to carry on the synthesis of endocuticle at the reduced rate characteristic of the postmolt period.

We have taken a closer look at epithelial RNA to determine the rates of synthesis and long-term stability of the ribosomes. After a single injection of tritiated uridine into intermolt animals, the ribosomes are labeled rapidly. The half-maximum of specific activity in 28 S RNA is reached in about 12 hours, and the maximum a day or so later. Although 95% of the injected radioactivity has disappeared from the hemolymph within 12 hours, the specific activity of the ribosomes remains constant for several weeks. If we inject 1000 times as much non-radioactive uridine as we had introduced into the animal with the first radioactive injection, the specific activity remains virtually unchanged; in other words, the radioactivity is not replaced by non-radioactive uridine. This means that the intermolt ribosomes are quite stable. After several weeks, however, the radioactivity begins to decrease, falling most rapidly 28 days after the initial injection. These results indicate that the life-time of these stable epithelial ribosomes is about four weeks in intermolt animals.

In comparison with the intermolt data, there are several similarities and one important difference in animals injected with tritiated uridine on the day of eyestalk removal, that is, at the onset of the premolt period. The epithelial ribosomes of such animals are labeled at about the same rate and to about the same extent, and have
Growth in *Geornicurus*

**TABLE 1. Comparative rates of incorporation in vivo of H\(^{3}\)-uridine into ribosomal (28 S) RNA of epithelium.**

<table>
<thead>
<tr>
<th>Stage in molt cycle</th>
<th>Rate of 28 S RNA synthesis (cpm/O.D. unit/8 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intermolt</td>
<td>2280</td>
</tr>
<tr>
<td>D(_0) early</td>
<td>1050</td>
</tr>
<tr>
<td>D(_0) late</td>
<td>215</td>
</tr>
<tr>
<td>D(_1)</td>
<td>365</td>
</tr>
<tr>
<td>D(_2)</td>
<td>70</td>
</tr>
</tbody>
</table>

life-times of about the same duration, as the intermolt ribosomes. The difference is seen when large amounts of non-radioactive uridine are injected 10-30 days after the initial injection; the crabs are now well into the premolt period, and the unlabeled uridine rapidly reduces the specific activity of the labeled ribosomes by 50-80\%. There are several possible interpretations of this result, one being that the amount of uridine ordinarily available to these animals is rate-limiting in the synthesis of ribosomal RNA. The cold uridine injected would then allow greatly enhanced synthesis which would dilute the pre-labeled RNA. There are other possible, if more complex, explanations involving changes in availability of RNA-precursor pools, and we cannot yet decide among alternatives. What is certain at this point is that the pattern and activity of RNA synthesis is changing as the premolt period progresses. This point is clearly seen when the rates of incorporation of radioactive uridine into ribosomal RNA at various times in the premolt period are compared (Table 1). Early in the premolt period, at the time limb buds are being regenerated and gastroliths are forming (stage D\(_0\)), the rate of incorporation is substantially the same as during the intermolt period. As the premolt period progresses, the rate has decreased considerably. By stage D\(_0\), after the exocuticle has been synthesized, the rate of incorporation has fallen off to less than 10\% of the intermolt level (Skinner, 1966).

To summarize the macromolecular changes in epithelium, we have seen that the enhanced synthesis of protein which occurs with growth of new cells and secretion of epi- and exocuticles is preceded by an increased amount of ribosomal RNA, which is lost later in the premolt period. Ribosomal RNA is stable for weeks, and, since we have not yet observed a time in the premolt period when the rate of RNA synthesis is enhanced, it is possible that the increased content of RNA seen in premolt is in fact due to a reduced rate of breakdown of ribosomes. The subsequent fall in RNA content is clearly associated with a markedly reduced rate of synthesis.

**SOMATIC MUSCLE**

The third tissue examined is somatic muscle from the chela and, in some experiments, from regenerating chela and walking legs. As before, I should like to define the growth period first. In 33 intermolt animals, we found that the total amount of muscle which can be removed from a chela is closely related to the external dimensions of the chela. From careful measurements of a claw, we can predict the intermolt muscle mass to within about 10\%. When we apply this calculation to chelae of premolt animals, the muscle mass actually found is consistently less than the amount expected in intermolt claws of similar size. By 10 days after eyestalk removal, the amount of recoverable muscle is detectably less than normal; two or three weeks later, it reaches a minimum value of 50-60\% of the intermolt mass, and remains reduced until about two weeks after eclosis. This is not due simply to loss of water, since there is no difference in the fractional dry weight or protein content of the intermolt and premolt muscle (Table 2). Hence, there is breakdown of a third to a half of the total muscle in a chela prior

**TABLE 2. Protein and RNA content of somatic muscle.**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Protein g/g wet weight</th>
<th>Protein g/g dry weight</th>
<th>RNA mg/g protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intermolt</td>
<td>0.177</td>
<td>0.93</td>
<td>7.2 ± 0.4</td>
</tr>
<tr>
<td>Premolt</td>
<td>0.179</td>
<td>0.94</td>
<td>10.2 ± 0.7</td>
</tr>
<tr>
<td>Postmolt</td>
<td>0.124</td>
<td>0.89</td>
<td>10.7 ± 0.5</td>
</tr>
</tbody>
</table>
MUSCLE

FIG. 5. The rate of incorporation in vivo of radioactive leucine into protein isolated from somatic muscle at various stages of the molt cycle. Redrawn from Skinner, 1965.

to ecdysis; this muscle is replaced and new muscle for the enlarged chela is synthesized late in the postmolt period.

Knowing that muscular growth is a postmolt phenomenon, we were surprised to find that the rate of incorporation of amino acids into muscle proteins shows two peaks (Fig. 5). The intermolt rate is very low, and increases six-fold in the premolt period. The rate then decreases again, and reaches a second maximum several weeks after ecdysis. The second maximum is readily understood, since it comes at a time when new tissue is being made. The first maximum, however, comes at a time when there is, in fact, a breakdown of tissue. We do not yet know the nature of the proteins being made at this time in the premolt period. From the work of N. O. Kaplan (personal communication) we know that an isomeric form of lactic dehydrogenase appears in the tissues of a number of crustaceans during the premolt period. It is quite possible that the high rate of protein synthesis in premolt muscles reflects a specific premolt change in enzyme composition of muscle, although the physiological significance of that change is unknown.

The amount of RNA in muscle is low (Table 2; Skinner, 1965). It increases about 40 per cent in the premolt period and remains elevated through ecdysis. It has been suggested (Wyatt and Linzen, 1965) that the marked increases in isolable RNA seen one day after injury of pupae of the saturnid, Antheraea pernyi (Barth, et al., 1945), may be due to changes in RNA binding to protein (or other cell fractions). Our data on the RNA content of muscle were based on orcinol analyses of acidhydrolyzed preparations, a method which purports to extract total RNA (Schneider, 1945). It is unlikely that differences in the binding of RNA would be stable to the strong acid used in this method. Phenol extractions of pre-, postmolt, and regenerating muscle also yield larger amounts of RNA on a basis of weight than are found in intermolt muscle (Skinner, 1966).

In other words, over one complete molting period, there is a net increase of RNA within an increased muscle mass inside an enlarged chela; the net increase of RNA occurs in the premolt period, while the net synthesis of muscle protein does not occur until several weeks after ecdysis.

The marked temporal separation between net RNA synthesis and net protein synthesis is seen most dramatically in the regenerating limb. In a single premolt period, Gecarcinus is able to regenerate a walking leg of almost the exact external dimensions as its normal opposite. The concentration of RNA in the regenerating limb bud a few days before ecdysis is 11 times more than the RNA concentration in muscle from a normal non-regenerating limb (Table 3). The day after ecdysis, the “regenerate” limb contains only one-sixth as much muscle as a normal “non-regenerate” limb of the same size. By the time the

<table>
<thead>
<tr>
<th>Time of sample</th>
<th>Material analyzed</th>
<th>Ratio of regenerated limb to control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day before ecdysis</td>
<td>RNA/g muscle</td>
<td>11.0</td>
</tr>
<tr>
<td>1 day after ecdysis</td>
<td>Total muscle/merus</td>
<td>&lt;0.16</td>
</tr>
<tr>
<td>Intermolt (membranous layer of exoskeleton fully formed)</td>
<td>Total muscle/merus</td>
<td>0.90</td>
</tr>
</tbody>
</table>
animal has entered the intermolt period, the amount of muscle is the same in both limbs. Here again, RNA is synthesized before ecdysis and growth of tissue occurs after ecdysis.

It was this observation which first prompted us to look at the stability of muscle ribosomes. The pattern of labeling of muscle 28 S RNA following a single injection of tritiated uridine on the day of eyestalk removal is similar to that of epithelial ribosomal RNA, except that muscle ribosomes are labeled much more slowly. In muscle, maximum specific activity is not reached for 6-10 days after injection. Once labeled, however, there is no significant loss of specific activity 40 days after the injection, and even the injection of large amounts of non-radioactive uridine at various times failed to chase the radioactive label out of these ribosomes. This great stability is, of course, what must be expected if the formation of ribosomes and the synthesis of muscle protein are separated by a period of weeks or months as appears to be the case in Gecarcinus.

**SUMMARY**

We can now turn to the final question: do all of these changes show the same pattern in time in all three tissues? In the premolt animal we found an increase in RNA synthesis in all tissues examined. This increase in ribosomal RNA is followed by a marked increase in the rate of protein synthesis whether or not the tissue is growing. After these generally parallel beginnings in the three tissues, there is a marked difference in their subsequent behavior. Ribosomal RNA of the midgut gland and epithelium decreases after the period of maximal protein synthesis just before ecdysis. However, ribosomes of somatic muscle appear to be conserved through ecdysis to be used in the postmolt replacement and growth of new tissue.

A considerable separation in time between the synthesis of RNA and the synthesis of protein has also been described in several other biological systems. Mature, unfertilized, echinoderm eggs contain all the ribosomal, messenger, and transfer RNA necessary for synthesis of protein from the time of fertilization up through the blastula stage. The unfertilized eggs with their full complement of RNA may remain in the ovary for weeks or months, ready at any moment to be discharged and fertilized, and to begin the rapid protein synthesis characteristic of their development. At the time of gastrulation, new RNA is needed and must be synthesized if the zygote is to develop normally (Nemer, 1963; Gross, et al., 1964). The same sequence of utilizing old and then, during gastrulation, new RNA has been described by Brown and Caston (1962) in amphibians.

Our experiments to date lead to a number of questions about the control of growth in Gecarcinus. Is ecdysone responsible for the synthesis of new RNA in all these tissues? What factors regulate the timing of protein synthesis after RNA synthesis? What control mechanism permits the ribosomes of midgut gland and epithelium to be broken down after the burst of protein synthesis seen in the premolt period, whereas the ribosomes of muscle are conserved for a second synthetic phase in the postmolt period?

Clearly, there remains much to be learned about the fine controls in the regulation of the different responses of the various tissues.

**REFERENCES**


Echalier, G. 1955. Rôle de l’organe Y dans le déterminisme de la mue de *Carcinides* (Carcinus) moenas L. (Crustacés Décapodes). Expériences


