Cytodifferentiation in the Accessory Glands of *Tenebrio molitor*

II. PATTERNS OF LEUCINE INCORPORATION IN THE TUBULAR GLANDS OF POST-ECDYSIAL ADULT MALES

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ABSTRACT The tubular accessory gland of male mealworm beetles undergoes rapid and progressive terminal differentiation in the 8-day period after ecdysis to the adult. Total protein and RNA content are maximal at five and eight days respectively. Rates of leucine incorporation rise gradually through the first four days and then increase abruptly in the 5- to 7-day interval. SDS-polyacrylamide gel electrophoresis demonstrates a variety of proteins; two classes with high mobility (Class A and B) appear prominent in homogenates of 5- to 8-day glands.

Double-label procedures show that as the glands mature, an increasing proportion of the total leucine incorporation passes into Class A and B proteins, until at eight days, Class A and B proteins account for 50% of the total for the gland. The relative incorporation into A vs. B also changes linearly over this interval. The developmental program of the tubular gland includes both a linearly biosynthetic increase in the proportion of differentiation-specific proteins and an abrupt change in the overall rates of leucine incorporation.

Cell differentiation involves both morphological specialization and underlying biochemical shifts in the patterns of protein and nucleic acid synthesis. Analysis of such differentiation is expedited by selection of convenient models in which both biochemistry and morphology are distinctive. We have chosen to investigate cytodifferentiation and its control in a set of useful models, the accessory reproductive organs of mealworm beetles. These include the spermatheca and its accessory gland in the female (Happ and Happ, '70, '75), and the tubular and bean-shaped accessory glands of the male (Gadzama, '72; Gadzama et al., '76). Each of these organs undergoes morphological terminal differentiation within the first week after ecdysis to the adult (Happ and Happ, '70, '75; Gadzama, '72; Gadzama et al., '77). In the three accessory glands, specific protein bands (defined by characteristic electrophoretic mobility) show increasingly higher rates of differential leucine incorporation in the first week of post-ecdysial adult life (present paper and Happ, unpublished). The biological environment for the differentiation, *Tenebrio molitor*, is a thoroughly studied insect which is quite amenable to experimental manipulation.

As a holometabolic insect, *Tenebrio* develops by cycles of apolysis and ecdysis: larval, pupal and adult. The structure and deposition of its body cuticle have been described at both light and electron microscopical levels (Wigglesworth, '48; Locke, '61; Delachambre, '70, '71). Mitotic activity and DNA synthesis in the epidermis have been described by Chase ('70). The changing ecdysone titres and critical periods for the body cuticle of the pupa and imago have been established by Delbecque and his co-workers (Delbecque et al., '75; Delbecque, '76). Numerous studies have defined other aspects of the endocrinological context for the cytodifferentiation of the accessory reproductive organs (e.g., Mordue, '65a,b,c; Menon, '70; Delachambre et al., '72; Krishnakumaran, '74; Provansal et al., '74; Lack and Happ, '76a,b). Especially important to our studies are the elegant surgical manipulations of Claude Huet which have demarcated many of the early events and the points of determina-

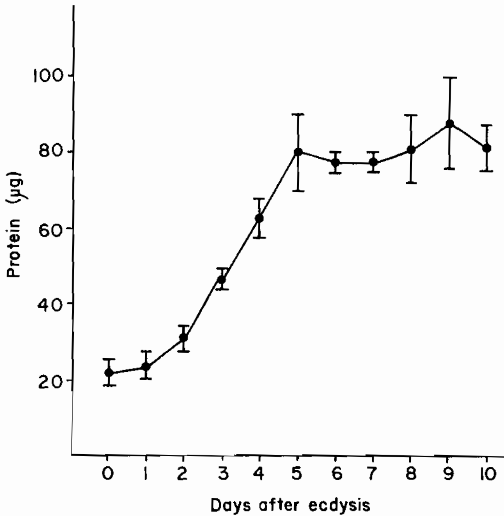

Fig. 1 Protein content for tubular glands determined by the Lowry procedure on aliquots from four to six gland pairs. BSA was the standard. At least four determinations were made at each age; the mean is plotted and the standard errors are indicated by the bars. In this and succeeding figures, data are expressed per gland pair.

tion for the accessory reproductive organs of *Tenebrio* in the last instar larva and the pupa (Huet, '65, '66, '68, '70, '72, '74, '75). Furthermore, the basic biochemical methodology for fractionation of subcellular components and for studying protein synthesis in *Tenebrio* has received considerable attention (Ilan and Ilan, '75 and references therein; Lassam et al., '76).

In the present paper, we will describe the changing patterns of protein and DNA content and of leucine incorporation in the tubular accessory glands of post-ecdysial adult males. As shown in the companion paper (Gadzama et al., '77), the morphological maturation of these glands is quite rapid in the first four days after the final ecdysis. Shortly thereafter, the males mate. The patterns of leucine incorporation suggest that progressive biochemical specialization coincides with the process of morphological terminal differentiation.

MATERIALS AND METHODS

Tenebrio molitor L is conveniently maintained in laboratory culture on a diet of Purina Startena with occasional additions of fruits and vegetables. Sexes were segregated as pupae; adults were collected as they ecdysed, and each age class was maintained separately at 26°C in an incubator. For the experiments described below, the accessory glands were exposed by dissection in *Tenebrio* saline (Butz, '57); the adhering fat was removed, and the paired tubular and bean-shaped accessory glands were separated from one another and from the remainder of the reproductive tract. Individual glands, or groups of several, were homogenized in an all-glass tissue grinder (2 ml total volume).

Protein determinations were by the Lowry procedure (Lowry et al., '52) with bovine serum albumin as the standard. RNA content was measured by an adaptation of the procedure of Raikow and Fristrom ('71). Glands were homogenized (approximately 5 pairs/ml) in Ringers and the protein was precipitated with the addition of 2 ml of cold 10% TCA (Trichloroacetic acid). After brief centrifugation (1,200 *g*, 10 minutes), the RNA in the supernatant was precipitated with 95% ethanol, and the precipitate was washed once more with 95% ethanol and then with diethyl ether. The dried precipitate was hydrolyzed in 0.3 N

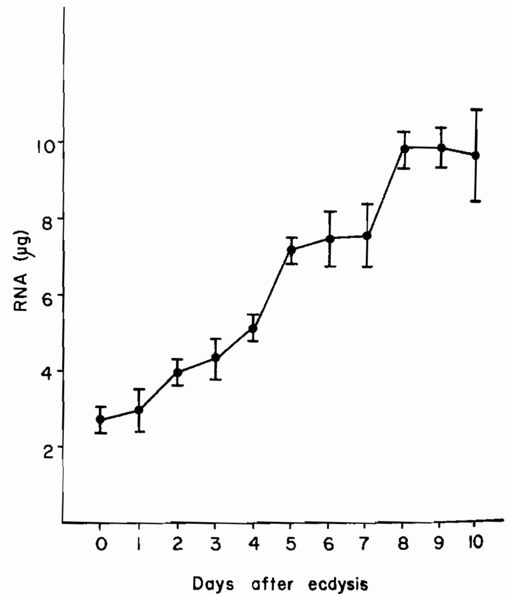

Fig. 2 RNA content for tubular gland pairs of various ages: Aliquots from homogenates of four to eight glands were examined at 260 and 280 nm as described in MATERIALS AND METHODS. At least three determinations were made at each age; means are plotted and standard errors are indicated by the bars.

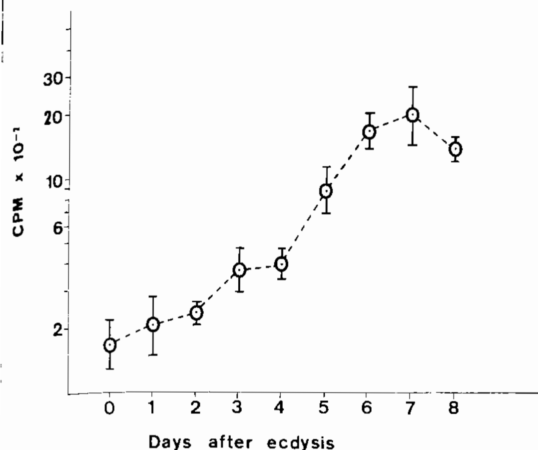

Fig. 3 ^{3}H incorporation into TCA-precipitable proteins of the tubular gland. Labelled leucine was injected and protein was isolated as described in MATERIALS AND METHODS. A minimum of eight gland pairs was examined for each day. The mean value and 95% confidence intervals are indicated.

NaOH (37°C, 1 hour). DNA and the remaining protein was precipitated with cold 1N $HClO_4$ and removed by centrifugation. The remaining supernatant was brought to pH 8.0 with Tris buffer, and the optical density was measured at 260 and 280 nm. Two standards (yeast RNA) were run in parallel with each set of experimentals; yields approximated 80%.

Electrophoresis was performed either on 10% urea-disc polyacrylamide gels (Kapitany and Zebrowski, '73) or on 7.5% SDS polyacrylamide gels (Weber and Osborne, '69). Before application to the SDS gels, samples were heated in solubilizing buffer (100°C, 10 minutes) (Paul et al., '72).

For studies of incorporation of radioactive leucine, beetles were removed from the incubator, affixed to microscope slides with wax, and briefly cooled to 3°C. Leucine was taken up in 2–4 μl of Ringers, injected into the abdomen, and the wound was sealed with wax (beeswax:paraffin, 1:1). Animals were subsequently maintained at room temperature.

Incorporation into TCA-precipitable proteins was measured by injecting 0.5 μC of tritiated leucine (3, 4, 5 ^{3}H; specific activity, 60 C/mM) or tritiated valine (2, 3, ^{3}H; specific activity 23 C/mM) allowing four hours of incorporation and then processing of individual glands was via the procedures of Kennell ('67). All TCA washes contained 10 mM of cold leucine or valine. The glass fiber discs were dried and counted in toluene-Eastman Dry Blend I cocktail on a Beckman LS-100 or on a Packard 3324 scintillation counter. Application of Bartlett's test to the raw data indicated that the values for subsequent days were heteroskedastic, i.e., the variances were significantly heterogeneous. A log transformation was performed to obtain homogeneity of variance before the 95% confidence intervals were calculated (Sokal and Rohlf, '69). Any datum point which differed by more than three standard deviations from the mean was omitted from the final calculations; this eliminated no more than one datum point for each day. In a preliminary experiment, valine incorporation was less than one-tenth of that for leucine; therefore valine injections were discontinued.

For studies of incorporation of leucine into specific bands (defined by electrophoretic mobility), ca. 3 μC of the ^{3}H-leucine or 1–1.5 μC of ^{14}C-leucine (uniformly labelled; specific activity 309 mC/mM) were injected as described above. After incubation (4–6 hours), the glands were removed, homogenized, and subjected to electrophoresis, usually on 7.5% SDS gels. For dou-

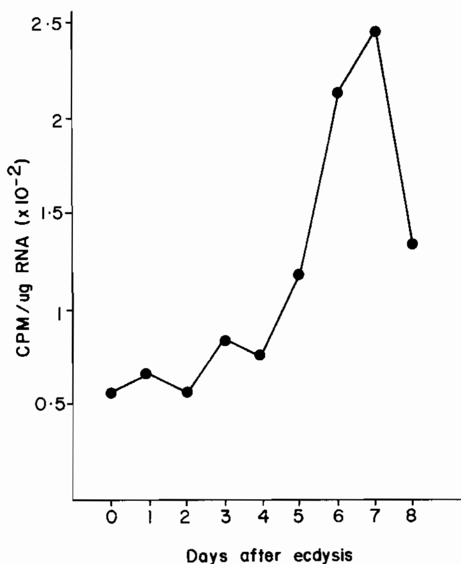

Fig. 4 ^{3}H-leucine incorporation/RNA ratios as a function of the age of the tubular gland. The leucine incorporation data are from figure 3, and the RNA content data are from figure 2.

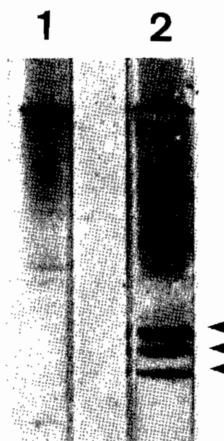

Fig. 5 Electrophoresis of tubular gland homogenates on urea-disc polyacrylamide gels. Gel 1 is homogenate of ten gland pairs from newly-ecdysed beetles, and gel 2 is homogenate of five gland pairs from beetles six days after ecdysis. The differentiation-specific proteins are indicated by the arrowheads.

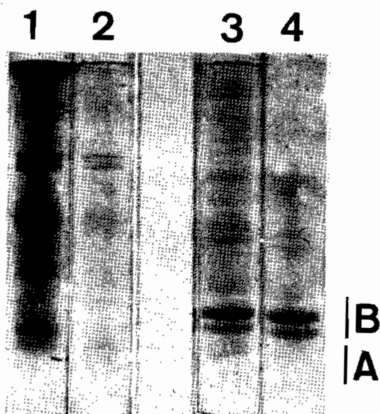

Fig. 6 Polyacrylamide gel electrophoresis of tubular gland homogenates on SDS gels. Homogenates from newly ecdysed beetles were applied to gels 1 and 2 and homogenates from 6-day beetles were applied to gels 3 and 4. Gel 1 was intentionally overloaded.

ble-label experiments, ^{3}H-leucine was injected into younger beetles, and ^{14}C-leucine into older beetles. The stained SDS gels were soaked for two hours in ethylene glycol:water (1:3) and frozen at -96°C. Transverse slices, cut on a BioRad Model 190 Gel Slicer, were solubilized in either Eastman Tissue Solubilizer or New England Nuclear Protosol. Both solubilizers were diluted 9:1 (solubilizer:water) and a 0.5 ml aliquot was added to each scintillation vial which contained a gel slice. The vial was heated at 50°C for two hours, allowed to cool, and 5 ml of cocktail was added. For the double-label experiments, counting time was 50 minutes/vial, using three windows of the Packard. In addition to the ^{3}H window (which had 11% ^{14}C crossover), two different ^{14}C windows were used.

The counts from the gel slices were analyzed by an adaptation of the Yund-Kafatos program (Yund et al., '71). In addition to the "dialect" modifications for the Colorado State University CDC 6400 computer, subroutines AXIS PENZ, SCALE, CURVE, and SET were substituted for the Harvard equivalents. In all cases, ratios of the counts in the two ^{14}C windows were constant throughout the slices of each gel, and thus quench was assumed to be constant throughout the gel.

RESULTS

The morphology of post-ecdysial maturation of the tubular accessory gland has been described in the companion paper (Gadzama et al., '77). At ecdysis, the secretory cells contain some rough endoplasmic reticulum but lack secretory vesicles. In the succeeding days, the cytomembrane becomes organized, and by four days after ecdysis, the cells are packed with secretory vesicles. Between four and six days, the cells increase markedly in size as they continue to accumulate secretory vesicles. The gland reaches maximum size and full morphological maturation at some point between six and eight days after ecdysis. At five to six days, male beetles mate readily and thus are apparently behaviorally and physiologically mature.

Protein and RNA in maturing tubular glands

Protein determinations of the total gland protein (cellular + luminal) show marked increases from 20 μg/gland pair at ecdysis to 80 μg/gland pair five days later (fig. 1). The increase from one to five days is essentially linear (Regression equation $Y = 14.5 \times + 5.25$; $r = 0.8688$). Over the fifth through the tenth day, the average protein content changes little.

The total RNA content of the tubular gland rises more gradually and plateaus only after the eighth day. The increase

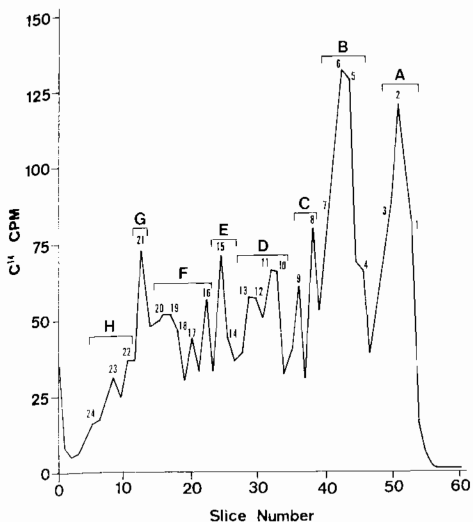

Fig. 7 ^{14}C-leucine incorporation into tubular gland homogenates from two male beetles, six days after adult ecdysis 1.0 μC injection, 50-minute count.

from the day of ecdysis (2.5 μg/gland pair) to eight days later (10 μg/gland pair) fits a linear regression model ($Y = 0.87 \times +2.31$, $r = 0.8712$). Although the protein content reaches its maximum value at five days when the gland volume is maximal, RNA content continues to increase for several days thereafter.

^{3}H-leucine incorporation into proteins

Over the first four to five days after ecdysis, gradually increasing amounts of injected ^{3}H-leucine were incorporated into TCA-precipitable proteins from gland homogenates (fig. 3). Between four and six days, the rate of incorporation increased sharply, and reached maximum at seven days (fig. 3).

The sharp increase in leucine incorporation is even more evident when leucine incorporation/glandular RNA ratios are plotted for each day (fig. 4). The incorporation/RNA ratios show a dramatic increase between four and six days.

Gel electrophoresis of tubular gland proteins

Proteins of the tubular gland can be readily separated by polyacrylamide gel electrophoresis. With either urea-disc or SDS techniques, 12 to 20 bands were clearly stained by coomassie blue. Comparison of homogenates of glands from newly ecdysed beetles with those from 5- to 8-day-old males revealed clear differences, especially in proteins with high mobilities in both electrophoretic systems (figs. 5, 6). On the basis of comparison with standards in the SDS techniques, the molecular weights

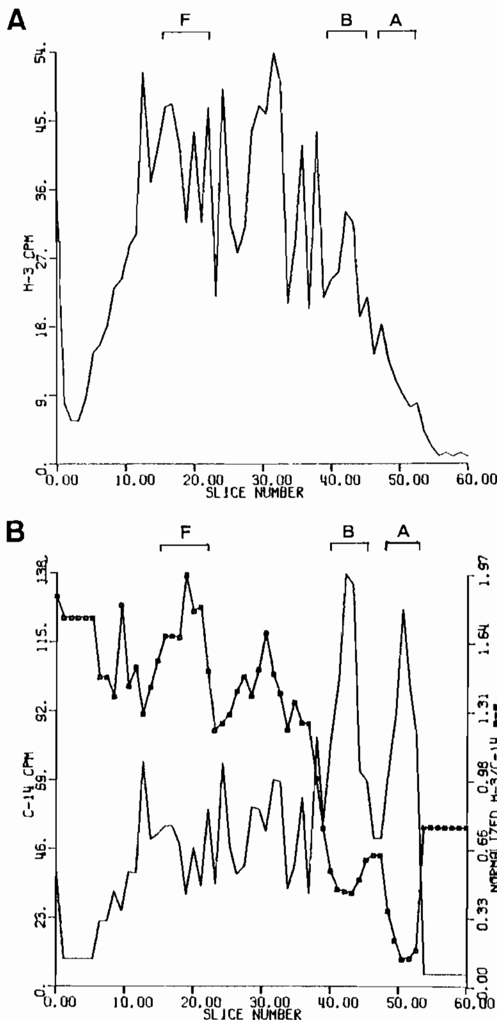

Fig. 8 Leucine incorporation into tubular gland proteins, separated by SDS-polyacrylamide gel electrophoresis. Zero-day beetles were injected with ^{3}H-leucine and 6-day beetles were injected with ^{14}C-leucine. Aliquots from the two tubular gland homogenates were combined and simultaneously run on the same gel. A shows the 0-day incorporation; B shows the 6-day incorporation and also the ratio of ^{3}H/^{14}C. Low ratios indicate higher relative incorporation into 6-day beetles.

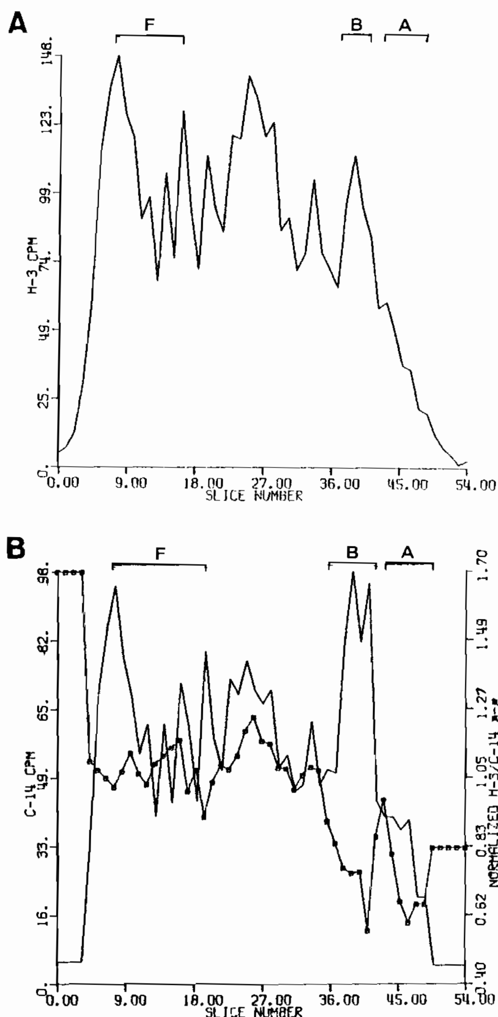

Fig. 9 Leucine incorporation into tubular gland proteins as in figure 8 A shows 0-day incorporation (^3H) and B shows the 2-day incorporation (^{14}C) and also the ratio of $^3H/^{14}C$.

of Classes A and B are estimated to be 12–14,000 and 15–18,000 respectively.

Leucine incorporation into protein bands

As described in MATERIALS AND METHODS, ^3H- and ^{14}C-leucine were injected into beetles of various ages; and four to five hours were allowed for incorporation of the leucine into the glandular proteins. The tubular glands were removed, homogenized, subjected to electrophoresis and the gels were sliced for scintillation counting. Leucine incorporation can be detected in at least 24 distinguishable bands (fig. 7). These 24 bands can be divided into several classes, A–H, as shown in figure 7. It proved difficult to identify unambiguously particular bands from newly-ecdysed beetles on the basis of their mobilities: markers were necessary for reference to avoid the ambiguity. Our marker was a tubular gland homogenate from an older beetle which had been injected with leucine labeled with another radioisotope.

As a rule for the double-label experiments, ^3H-leucine was injected into younger beetles and ^{14}C-leucine into the older ones. After allowing time for incorporation, we mixed aliquots from homogenates of the younger and of the older tubular glands and applied the combined double-labeled homogenates to a single 7.5% SDS polyacrylamide gel. The results from these experiments are shown in figures 8 to 11.

The patterns of leucine incorporation in tubular glands from newly ecdysed males are compared with those from 6-day males in figure 8. Inspection of these data reveals marked differences in the $^3H/^{14}C$ ratios for the various bands, indicating that the relative emphasis on the alternative biosynthetic products changes markedly as the tubular glands mature. The most prominent Class A (Bands 1–3) and Class B (Bands 4–7), have $^3H/^{14}C$ ratios which are much less than 1. Relative to the total leucine incorporation into tubular gland proteins, these two groups took up a much higher proportion of the leucine in six day males (^{14}C-leucine) than in newly ecdysed males (^3H-leucine). Examination of the other classes of proteins suggests that Classes D and F showed higher incorporation in the glands, from younger animals, while relative C, E, and G incorporation is fairly constant at both ages. The small peaks of Class H are not well resolved by this gel system and will not be considered further.

Figure 9 presents a comparison of glands from a newly ecdysed beetle (^3H-leucine) with those from beetles two days older (^{14}C-leucine). Once again, the $^3H/^{14}C$ ratios show that the relative rates of leucine incorporation differ between the two stages of tubular gland maturation. Classes A and B are favored in older beetles, and to a lesser extent, Class F is differentially emphasized in younger beetles.

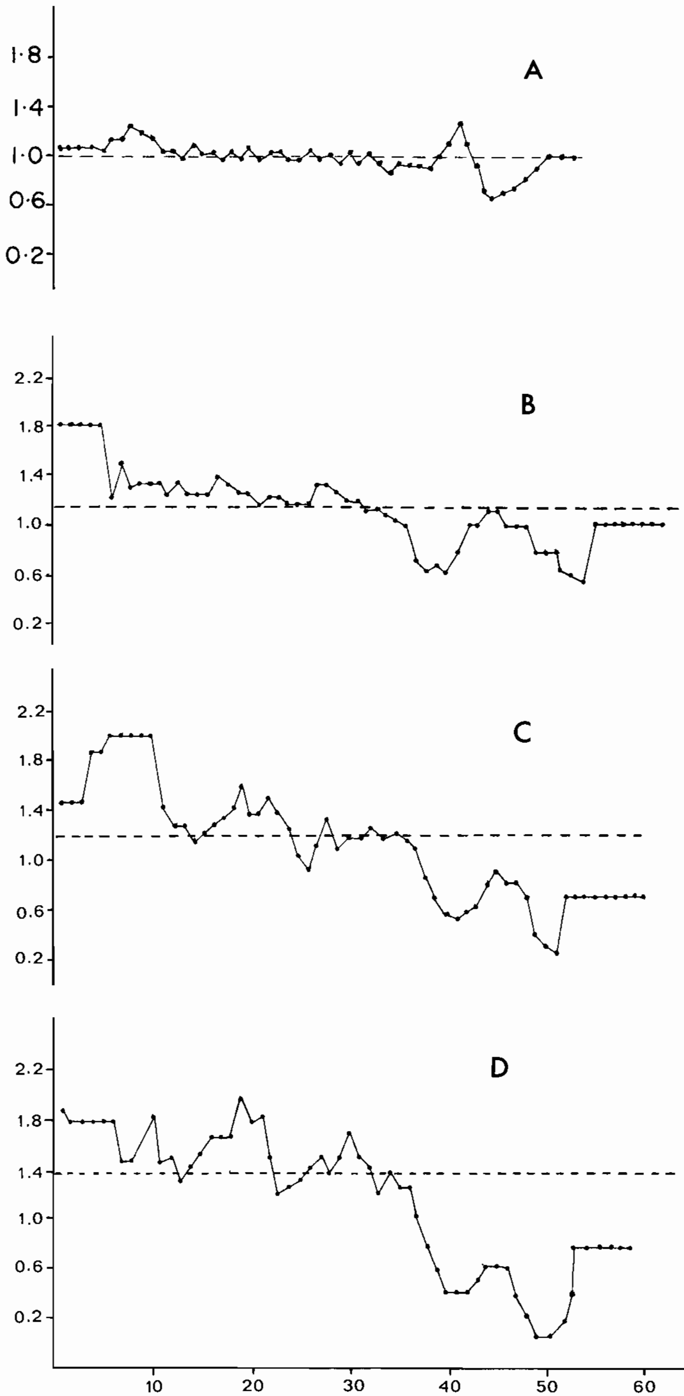

Fig. 10 $^3H/^{14}C$ ratio for leucine incorporation into proteins of tubular gland homogenates subjected to electrophoresis on 7.5% SDS polyacrylamide gels. In all cases, the younger glands were labelled with ^3H and the older ones with ^{14}C; thus values below the median ratio (indicated by the dashed line) show higher incorporation in the older glands of combined sample. A: 6 vs. 8 days; B; 4 vs. 6 days; C: 2 vs. 8 days; D: 0 vs. 6 days.

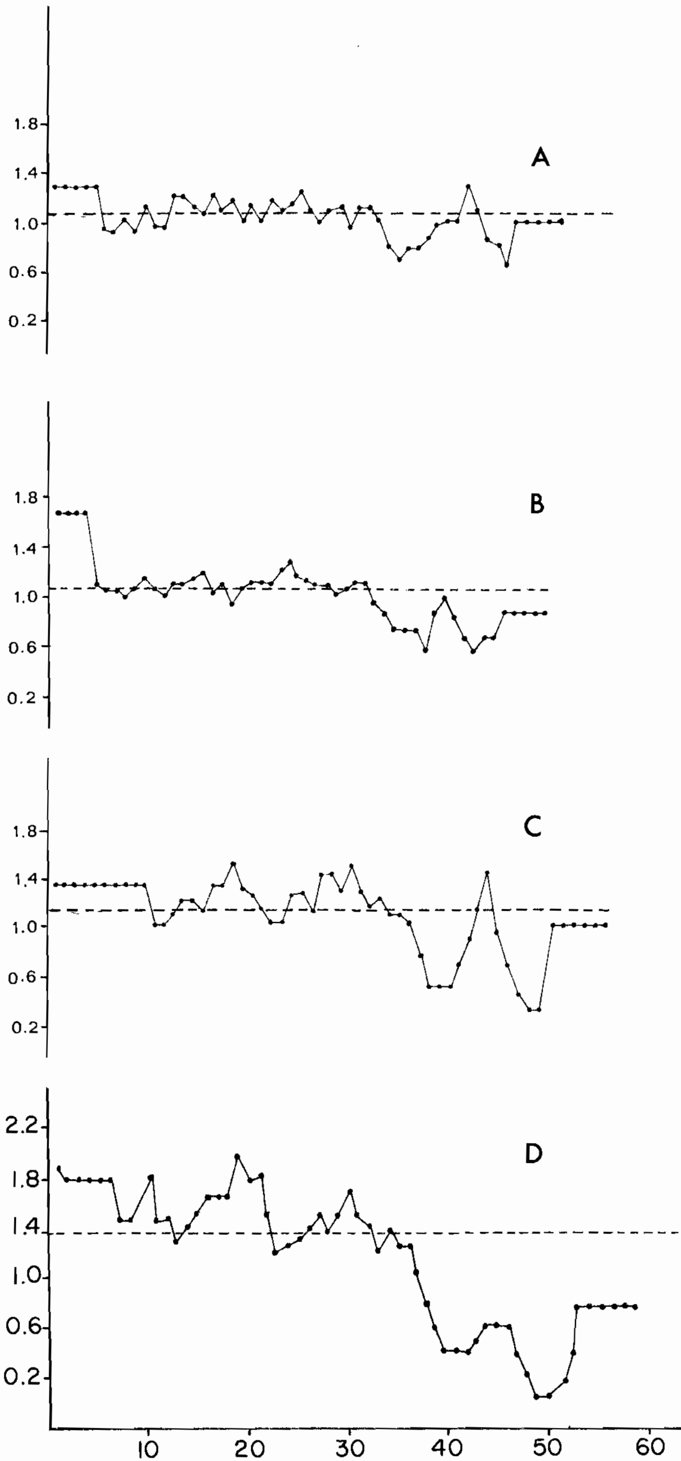

Fig. 11 $^3H/^{14}C$ ratios as in figure 10. A: 2 vs. 4 days; B: 0 vs. 2 days; C: 0 vs. 4 days; D: 0 vs. 6 days.

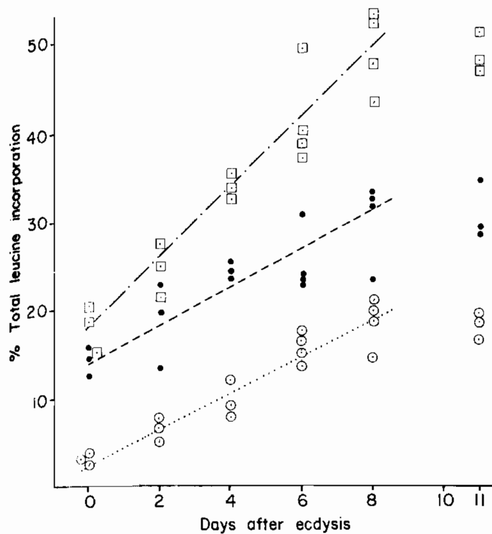

Fig. 12 Percentage of total leucine incorporation into Class A and Class B proteins over the first 11 days after ecdysis. Class A [O]; Class B [●]; Classes A + B [□]. The regression equations for the first eight days are: Class A, Y = 1.96X + 2.97, (r = 0.95); Class B, Y = 2.03X + 14.52, (r = 0.90); Classes A + B, Y = 3.94X + 17.65, (r = 0.96).

The progressive nature of changes in leucine incorporation can be seen better when the $^{3}H/^{14}C$ ratios of several paired days are examined in parallel. Figure 10 presents the comparison of glands from beetles six days after ecdysis with those from beetles zero, two, four, and eight days after ecdysis. Both Class A and B have disparate rates of leucine incorporation in the 0/6 comparison (fig. 10D), somewhat higher rates in the 2/6 comparison (fig. 10C), and the greatest similarities occur when the age difference is only two days (figs. 10A,B). Analogous comparisons of 2/4, 0/2, and 0/4 days yield a complementary pattern (figs. 11A–D). In aggregate, these ratio plots show that shifts in the patterns of leucine incorporation, especially leucine incorporation into Classes A and B continue to occur for eight days after ecdysis.

The two minima in $^{3}H/^{14}C$ ratios precisely define the A and B protein regions. From the Yund-Kafatos computer printout, we were able to sum the percent of total leucine incorporation which was characteristic of each gel and each isotope in Class A and Class B. These percentage data are shown in figure 11 for Classes A and B, and also for their combined leucine incorporation as a function of beetle age. The increase in percentage of leucine incorporation is linear from day 0 to day 8, and the rate of increase (slope) for A and B is identical. A linear regression model fits these data very well (fig. 12).

The relative incorporation into A vs. B is not constant: for A the increase is 5-fold whereas for B it is approximately 2-fold. When the B/A ratios are calculated and plotted as a function of beetle age, a linear regression model is also applicable (fig. 13).

Both the percentage of leucine incorporation and the B/A ratios provide convenient quantitative indices of the stage of differentiation in the tubular accessory gland. It should be emphasized, especially for the data from newly ecdysed beetles, that it is imperative that the homogenates be run simultaneously with a differently labeled older homogenate; otherwise the A region cannot be accurately defined in the gel.

In vivo incorporation into tubular gland proteins might stem either from protein synthesis, endogenous to the tubular glands, or from protein synthesis elsewhere in the animal and absorption of the protein products by the tubular gland. At least for Class A and B proteins, the tubu-

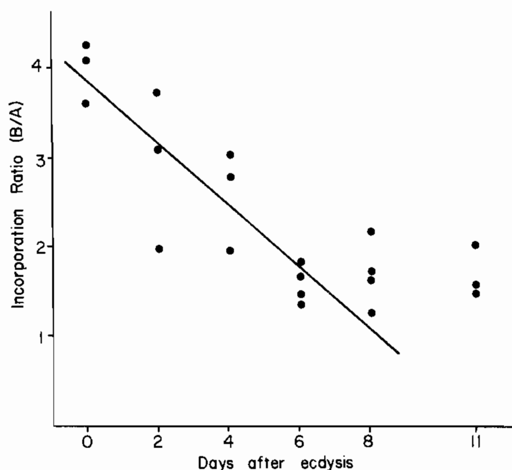

Fig. 13 Ratio of leucine incorporation into Class B proteins relative to Class A proteins as a function of days after ecdysis. The regression equation for the first six days is: Y = −0.3423X + 3.82; r = −0.8742.

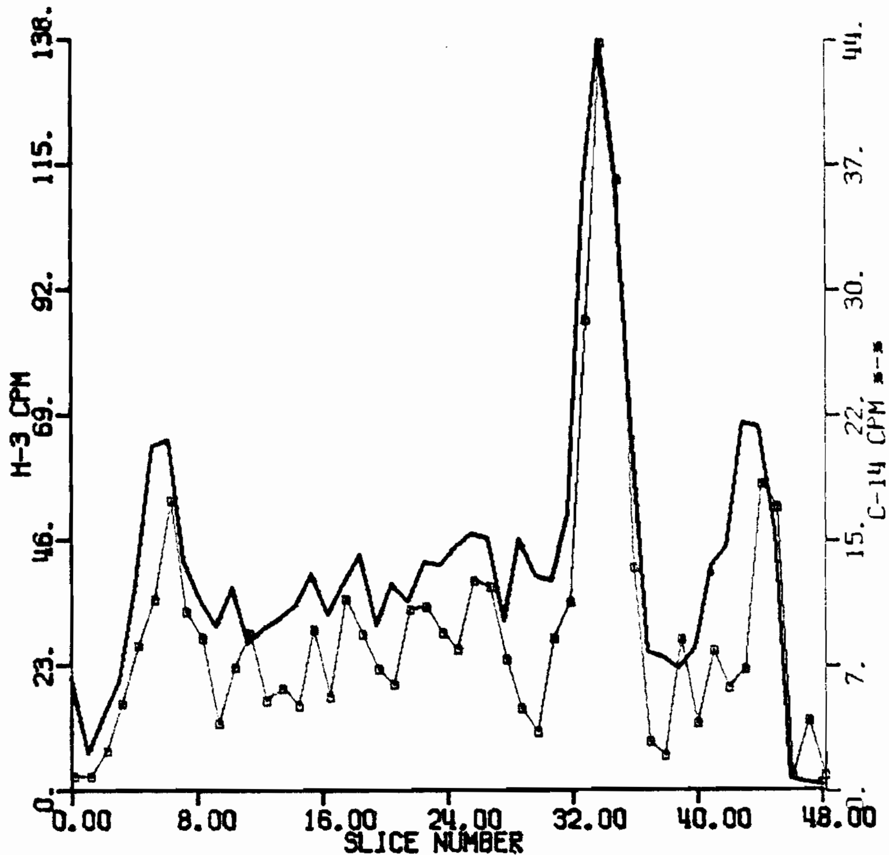

Fig. 14 Leucine incorporation in vivo (^{14}C) and in vitro (^{3}H). In vivo labelling with 6-day males; in vitro labelling by incubation of three glands from 7-day males in 0.1 ml Grace's medium (GIBCO, without addition of haemolymph or albumin) plus 1 μC of ^{3}H-leucine. 1,970 ^{3}H CPM and 460 ^{14}C CPM on 7.5% SDS gel.

lar gland alone is sufficient to support leucine incorporation in vitro (fig. 14).

DISCUSSION

Differentiation in the tubular gland

As the morphological terminal differentiation of the tubular accessory gland proceeds (Gadzama et al., '77), the biochemical machinery becomes increasingly specialized for the production of Class A and Class B proteins. Over the first four days of the post-ecdysial terminal differentiation, gradual increases in protein content, in the RNA content, in the rate of leucine incorporation, and in the ratio of leucine incorporation: RNA content parallel the gradual increases in gland volume, in the thickness of the secretary epithelium, and in the accumulation of secretory vesicles in the apical regions of the secretory cells (Gadzama et al., '77).

Between four and seven days after ecdysis, when the morphological secretory machinery is already qualitatively mature, a rapid change occurs in certain quantitative aspects of the terminal differentiation: the rate of leucine uptake accelerates sharply; the protein content reaches its maximum, and the gland grows to full volume.

As shown by the leucine incorporation into specific electrophoretic bands, a variety of proteins are manufactured throughout the post-ecdysial period of maturation. These proteins fall conveniently into two groups: Class A and B proteins which are differentiation-specific, and Class C proteins which are made at relatively lower or similar rates in older glands than in younger ones. Of some interest is the fact that the changing pattern of protein synthesis over this post-ecdysial period is

simple linear function of time, such that at eight days the synthesis of differentiation-specific proteins accounts for half of the total leucine incorporation. Perhaps equally important is the fact that slopes of the two curves for percentage leucine incorporation of A and B proteins are identical, suggesting co-ordinate controls. But whatever the relationship between the two groups, the percentage of leucine incorporation and the B/A ratio of percentages provide two quantitative indices of the extent of ongoing terminal differentiation in the tubular accessory gland.

Accessory glands as examples of cell maturation

Because developing exocrine secretory systems often exhibit a progressively increasing emphasis on biosynthesis of restricted populations of protein species, they have proved to be especially convenient materials for studies of general properties. The numerous model systems range from chick oviduct (Palmiter et al., '71) and rat pancreas (Rutter et al., '68) to the silk gland of moths (Suzuki and Brown, '72) and the insect ovarian follicle (Paul et al., '72; Paul and Kafatos, '75; Kafatos, '75; Petri et al., '76). The accessory glands of many male insects offer equally favorable opportunities for general studies of cell differentiation, as well as a variety of challenging but more parochial problems for insect endocrinology, reproductive physiology, and biochemistry.

Morphological studies of terminal differentiation in male accessory glands have been reviewed in the companion paper (Gadzama et al., '77). Of greater relevance to the present paper are the more biochemical studies, particularly those using house crickets (*Acheta domesticus*) and migratory locusts (*Locusta migratoria*). In both of these insects, the male accessory glands are closely associated with spermatophore formation. Although the accessory gland is present over several instars of these hemimetabolous insects, it does not become fully functional until several days after the final ecdysis to the adult.

In the accessory glands of *Acheta*, the protein products are many and diverse. Sucrose gradient centrifugation, SDS polyacrylamide gel electrophoresis, and anodal disc polyacrylamide gel electrophoresis demonstrate molecular weights from 10,000 to 100,000 daltons and a variety of charge properties (Kaulenas et al., '75). The *Acheta* gland is composed of several hundred tubules of differing lengths: the poly-tubular gland can be divided into six regions, and each region apparently synthesizes a different population of proteins (Kaulenas, '76). Similar regional differences in synthetic activity of the multi-tubular accessory gland of a desert locust (*Schistocerca*) were reported by Ohdiambo ('69) on the basis of phase-contrast and histochemical examination of the exudates.

In the accessory gland of *Locusta*, the protein content increases linearly for the first fourteen days after ecdysis. Incorporation of labelled glycine by the accessory gland is maximal at seven days, and then decreases. Removal of the corpora allata prevents growth of the accessory gland and reduces the incorporation of the label to its proteins (Gillott and Friedel, '76).

Proteins of accessory glands

Protein products of male accessory glands of insects play three major roles: (1) as components of the seminal plasma itself, (2) as structural proteins of the spermatophore, and (3) as regulatory signals which are transferred to the female and act upon her physiological state (Englemann, '70; de Wilde and De Loof, '73; Hinton, '74; Leopold, '76). For *Tenebrio*, both amino acid analyses and immunochemical evidence demonstrate strong similarities between the bean-shaped accessory gland and the spermatophore (Frenk and Happ, '76). However, the precise function of the tubular gland secretion is not known.

Surprisingly little is known about the biochemical characteristics of proteins produced by invertebrate accessory glands. With four major exceptions (the seminal proteins of barnacles [Barnes and Blastock, '74], the proteins of the accessory gland and spermatophore of an octopus [Mann, '70], the paragonial substances of *Drosophila* [Baumann, '74], and the sperm activator of saturniid moths [Shepherd, '74a,b, '75]) available biochemical data are largely restricted to displays of protein bands on polyacrylamide gels. The histochemical data suggest molecular diversity (Adiyodi and Adiyodi, '75), but such histochemical information lacks biochemical precision. When one considers the importance of seminal and paraseminal pro-

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