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PATTERNS OF LEUCINE INCORPORATION IN THE SPERMATHECAL ACCESSORY GLANDS OF THE POST-ECDYSIAL ADULT FEMALE *TENEBRIO MOLITOR*

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Abstract—The spermathecal accessory glands of female mealworm beetles become fully active by six days after pupal–adult ecdysis. Coincident with the morphological maturation, increases occur in protein and RNA content and in total leucine incorporation. Three classes of differentiation-specific proteins are increasingly prominent and show heightened rates of leucine incorporation by the sixth day.

INTRODUCTION

REPRODUCTIVE maturation involves not only the gonads but also a variety of secondary structures; the transport systems (e.g. oviducts and vasa deferentia), the storage areas (e.g. egg sacs, seminal vesicles, spermathecae) and the diverse accessory glands whose products lubricate, nourish, activate and/or protect eggs and sperm as well as act as signals from male to female (LEOPOLD, 1976). In many holometabolous insects, the accessory glands undergo primary growth and morphogenesis in the pupa and pharate adult. This investigation is an attempt to analyze the processes of accessory gland differentiation and their controls in mealworm beetles, *Tenebrio molitor*.

Both male and female *Tenebrio* have distinct reproductive accessory glands. Those of the male, the tubular and bean-shaped accessory glands (JONES, 1967; GADZAMA, 1971; POELS, 1972a and b; GERBER, 1976; GADZAMA *et al.*, 1977) are paired, derived from mesodermal rudiments (HUET, 1966) and lack a cuticular lining, while the spermatheca and spermathecal accessory gland of the female are unpaired, ectodermal in origin (HUET, 1974) and lined with cuticle (HAPP and HAPP, 1970, 1975).

The spermathecal accessory gland of female *Tenebrio* is an elongate cylinder of secretory epithelium surrounding an axial cuticular duct. Each of the secretory cells is found in an organule (LAWRENCE, 1966; KUHN, 1971; KAFATOS, 1972) with an export system of fine cuticular-ductules which empty into the axial duct. The ultrastructure of the adult gland has been reported (HAPP and HAPP, 1970) and the morphological aspects of organogenesis and cuticle deposition between larval–pupal and pupal–adult ecdysis have been examined (HAPP and HAPP, 1978). In the present paper, we report the results from a study of patterns of leucine incorporation into proteins of the SAG during post-ecdysial adult terminal differentiation.

MATERIALS AND METHODS

Mealworm cultures were maintained on a diet of Purina Startena and sexes were segregated at the pupal stage. Females were collected at adult ecdysis and maintained with other females of the same age at 26°C. Spermathecal accessory glands were exposed by dissection in *Tenebrio* saline (BUTZ, 1957) or in distilled water; they were cleaned of fat body and other adhering materials and homogenized in an all-glass grinder.

Protein determinations were by the Lowry procedure (LOWRY *et al.*, 1951) with bovine serum albumen as the standard. RNA content was measured by an adaptation of the procedure of RAIKOW and FRISTROM (1971). Glands were homogenized (approximately 5 glands/ml) in distilled water and the protein was precipitated with the addition of 2 ml of cold 10% trichloroacetic acid. After brief centrifugation (1100 **g**, 10 min), the RNA in the precipitate was washed with 95% ethanol, centrifuged again and washed with 95% diethyl ether. The dehydrated precipitate was hydrolyzed in 0.3 N NaOH (37°C, 1 hr). The DNA and protein were precipitated with 1 N $HClO_4$ and removed by centrifugation. The remaining RNA in the 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 experimental samples; yields approximated 80%.

Electrophoresis was performed on 5–20% disc polyacrylamide gels (ORNSTEIN, 1964; DAVIS, 1964) on 10% urea-disc polyacrylamide gels (KAPITANY and ZEBROWSKI, 1973) or on 7.5% SDS polyacrylamide gels (WEBER and OSBORNE, 1969). Before application to the SDS gels, samples were heated in solubilizing buffer (100°C, 10 min) (PAUL *et al.*, 1972).

For studies of incorporation of radioactive leucine, beetles were removed from the incubator, affixed to microscope slides with beeswax, and briefly cooled to 3°C. Leucine was taken up in 2–4 μl of Ringer's, injected into the abdomen and the

wound was sealed with wax (beeswax: paraffin, 1:1). Animals were subsequently maintained at room temperature during the period of incorporation.

Incorporation into TCA-precipitable proteins was measured by injecting 0.5 μCi of tritiated leucine (3, 4, 5, ^{3}H; specific activity, *ca.* 60 Ci/mM) or tritiated valine (2, 3, ^{3}H; specific activity, 23 Ci/nM), by allowing 4 hr for incorporation and then by processing the individual glands via the procedures of KENNELL (1967). All TCA washes contained 10 mM of cold leucine or valine. The glass fibre discs were dried, immersed in toluene–Eastman Dry Blend I medium and counted on a 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, 1969). 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 μCi of the ^{3}H-leucine or 1–1.5 μCi of ^{14}C-leucine (uniformly labelled; specific activity, 250–300 mCi/mM), were injected as described above. After incubation (4–6 hr) the glands were removed, homogenized and subjected to electrophoresis, usually on 7.5% SDS gels. For double-label experiments, ^{3}H-leucine was injected into younger beetles and ^{14}C-leucine into older beetles. The stained SDS gels were soaked for 2 hr in 25% ethylene glycol and frozen at −96°C. One millimeter transverse slices were each solubilized in 0.5 ml Protosol (9:1, solubilizer: water). Each sample containing a gel slice and tissue solubilizer was then heated at 50°C for 2 hr, allowed to cool and dissolved in 5 ml of scintillation medium. For the double-label experiments, counting time was 10–50 min/vial, using three windows of the counter. In addition to the ^{3}H window (which had 11–12% ^{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.*, 1971) as described by HAPP *et al.* (1977). 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.

RESULTS

At the time of pupal–adult ecdysis, the cuticular export system of the SAG has been completely formed and the apical surface of each secretory cell surrounds a perforated cuticular end apparatus which connects, via an efferent cuticular ductule, with the gland's axial duct. Within the cytoplasm of the secretory cells, most of the ribosomes are not associated with membranes, and the Golgi zones are small. Over the first four days after pupal–adult ecdysis, the cytoplasm becomes filled with rough endoplasmic reticulum and the Golgi zones enlarge. Secretory products, confined within membrane-bound vesicles, appear to move from the Golgi zones to the apical plasma membrane. Over the second, third and fourth days after ecdysis, the extracellular space between the apical plasma membrane and the end apparatus becomes progressively larger with successive additions of secretory product. At four days and older, a prominent central cavity of extracellular, secretion-filled space surrounds the end apparatus (HAPP and HAPP, 1970).

Protein and RNA content

Over the first 4–6 days after pupal–adult ecdysis, both protein and RNA content increase markedly. The total protein increases from *ca.* 10 μg/SAG to *ca.* 25 μg/SAG and thereafter remains fairly constant. The increase from one to six days fits a linear regression model quite well (Regression equation: $Y = 2.11X + 8.65$, $r = 0.72$); the RNA content rises in a similar manner (Regression equation: $Y = 0.51X + 1.0$, $r = 0.803$).

Proteins of the spermathecal accessory gland are readily displayed by polyacrylamide gel electrophoresis with both disc and SDS-techniques. A comparison of glands from newly-ecdysed females with those from older ones shows strong enhancement of the staining in particular regions.

With 5–7% disc gels, the staining enhancement is primarily in a fast moving band just behind the tracking dye. This prominent band stains well with both Alcian blue (for acidic polysaccharides) and Coomassie blue (for proteins). Increasing the percentage of acrylamide to 15–20% resolves the fast-moving band into several components. One of these moves with the tracking dye and stains heavily with Alcian blue but not with Coomassie blue. Several others move more slowly (in the mid-region of the

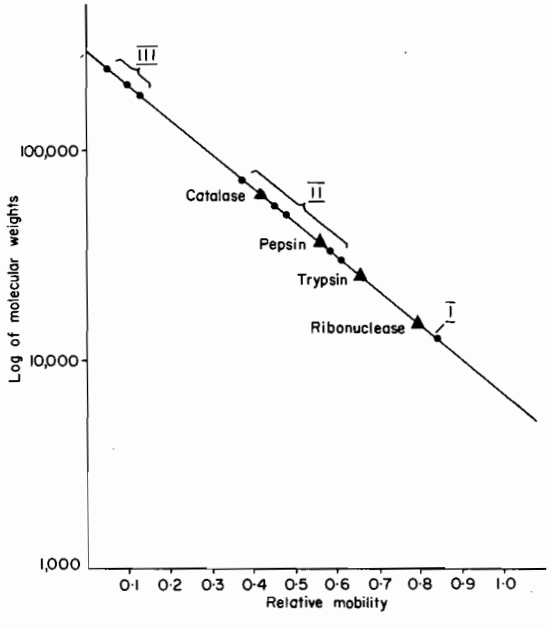

Fig. 1. Molecular weight standards (triangles) and the SAG proteins (solid dots) from Classes I, II and III as displayed on 7.5% SDS polyacrylamide gels.

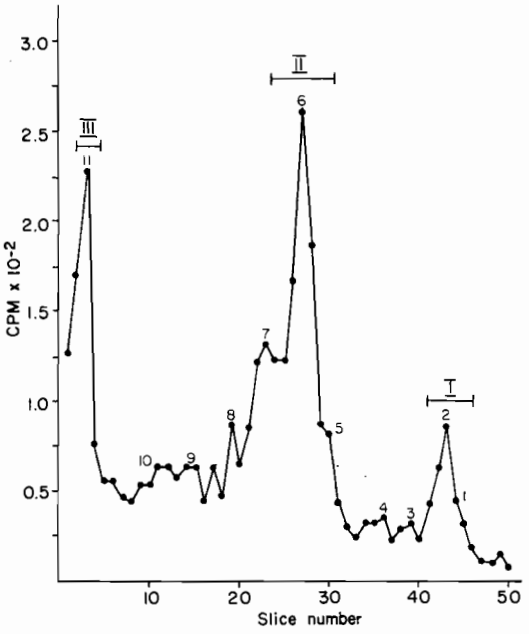

Fig. 2. ^{3}H-leucine incorporation into 6-day SAG, analyzed by SDS polyacrylamide gel electrophoresis.

gel) and stain strongly with Coomassie blue but only very lightly with Alcian blue. On urea-disc gels, the terminal band stains only with Alcian blue and the mid-region bands have no Alcianophila. According to our earlier histochemical data (HAPP and HAPP, 1970), the secretory products of the SAG are acidic glycoproteins. The electrophoretic evidence presented here suggests the polysaccharide-protein bonds are relatively weak and therefore not covalent. A similar conclusion was reached by JONES (1974).

SDS-polyacrylamide gel electrophoresis consistently yields 23–27 bands which stain with Coomassie blue. Three classes of differentiation-specific proteins, indicated in Fig. 1 as Classes I, II and III, correspond to mol. wt of 12,800, 30–65,000 and 180,000–220,000 respectively.

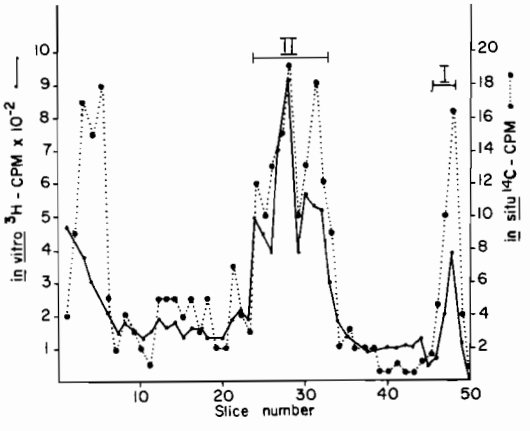

Fig. 3. Leucine incorporation *in situ* (^{14}C) and *in vitro* (^{3}H), after incubation in Grace's medium plus 1 μCi of ^{3}H-leucine.

Leucine incorporation into the SAG proteins

^{3}H-leucine incorporation into total TCA-precipitable proteins increased steadily over the first 6 days after pupal–adult ecdysis and was fairly constant for several days thereafter. Separation of the radioactive proteins upon SDS-polyacrylamide gels and determination of the radioactivity in each 1 mm slice revealed at least eleven consistently distinguishable bands (Fig. 2). Three groups of proteins, corresponding to Class I, II and III seen on the stained gels, were particularly prominent.

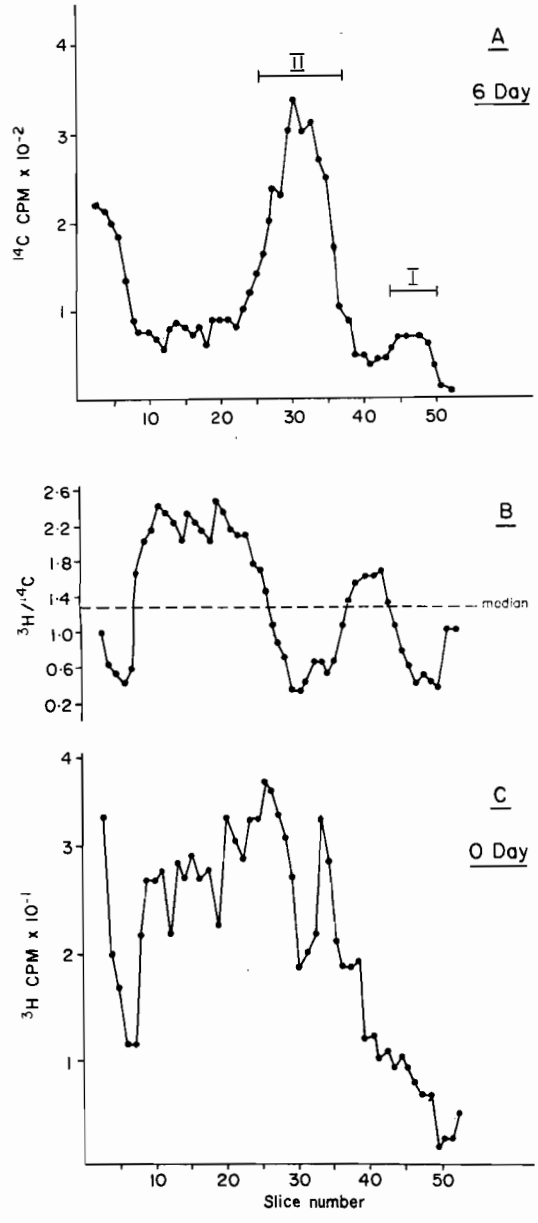

Fig. 4. Leucine incorporation into the SAG proteins. 0-Day beetles (after ecdysis) were injected with ^{3}H-leucine and 6 day beetles were injected with ^{14}C-leucine. Aliquots from the SAG homogenates of the two ages were combined and run on the same SDS gel. A shows the 6-day incorporation, C shows the 0-day incorporation, and B the ratio of ^{3}H/^{14}C. Low ratios in B indicate higher relative incorporation into the SAGs of older (6 day) beetles.

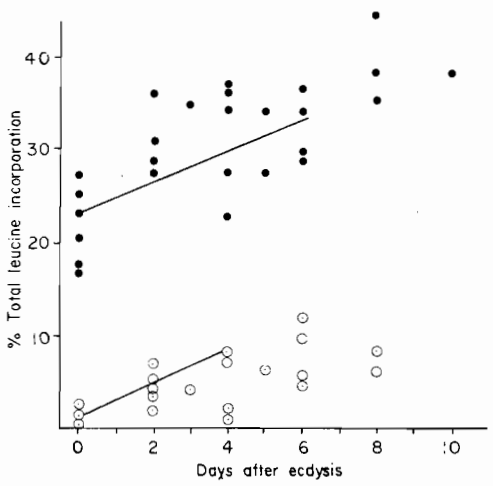

Fig. 5. Percentage of total leucine incorporation into Class I (○) and Class II (●) proteins over the first 8 days after ecdysis. The regression equations for Class I is $Y = 1.01X + 1.86$, $r = 0.72$ (0–4 days) and for Class II is $Y = 1.78X + 23$, $r = 0.67$ (0–6 days).

On the basis of the above *in situ* experiments, one cannot distinguish between synthesis of the differentiation-specific proteins within the SAG itself and mere absorption of such proteins from the haemolymph after synthesis in other tissues. To resolve this uncertainty, six SAGs were removed from female *Tenebrio* and incubated in Grace's medium supplemented with ^{3}H-leucine. Subsequent electrophoresis of the products showed an incorporation pattern very similar to the *in situ* experiments (Fig. 3). Thus, the differentiation-specific proteins of at least Classes I and II appeared to be synthesized within the SAG itself.

The increased incorporation of leucine into the Class I–III proteins can be seen clearly in gels with a double isotopic label. ^{3}H-leucine was injected into younger beetles (0, 2, 4 and 6 days) and ^{14}C leucine into older ones (6 and 8 days). The spermathecal glands were homogenized and applied simultaneously to the same gel. In Fig. 4, the results from an age comparison are shown. Increasing percentages of total leucine were incorporated into the Class I and II proteins as the beetles mature (Fig. 5). Similar increases probably occur in Class III proteins, but on the 7.5% gels the higher molecular weight proteins were often difficult to distinguish from material caught at the top of the gel.

DISCUSSION

In the tubular accessory gland (TAG) of male *Tenebrio*, as in the SAG, leucine incorporation into the differentiation-specific proteins and the total rate of leucine incorporation are both maximal at six days after pupal–adult ecdysis (HAPP *et al.*, 1978). Both of these rates offer useful indices for scoring the extent of differentiation. An additional feature of the maturation of the TAG is a progressive shift between the two classes of differentiation-specific proteins, until six days after ecdysis when the ratio of Class B to Class A is approximately 2. No such precisely quantitative pattern of interclass changes emerges from our SAG data. This lack of an evident pattern in the SAG may be due simply to interclass shifts within the several Class II proteins which are not consistently resolved from one another in the 1 mm slices.

Biochemical studies of maturation of the accessory glands in other insects involve both colleterial glands of the female, as in *Periplanta* (BODENSTEIN and SHAAYA, 1968) and glands of the male, as in *Melanopus* (GILLIOTT and FRIEDEL, 1976) and *Acheta* (KAULENAS, 1976). In these hemimetabolous orthopteroid insects, the progressive maturation accelerates sharply after ecdysis to the adult. In all three genera, the corpora allata are implicated in the regulation of the accessory gland secretion. In *Rhodnius*, *Schistocerca*, *Gromphocerus*, *Periplaneta*, the corpora allata control morphological maturation (WIGGLESWORTH, 1936; CANTACUZENE, 1967; HARTMANN, 1971; BLAINE and DIXON, 1973). Amongst the holometabolous insects, in *Calliphora*, the corpora allata apparently do not affect the male accessory glands (THOMSEN, 1943) while in *Danaus*, *Nymphalis* and *Leptinotarsa*, the corpora allata are involved in the recovery from reproductive diapause of adults (HERMAN, 1975; HERMAN and BENNET, 1975; DE LOOF and LAGASSE, 1972). The corpora allata have been implicated in the control of sex pheromone release in the post-ecdysial adult of *Tenebrio* (MENON, 1970), but juvenile hormone is not required for uptake of vitellogenins by the ovary (LAVERDURE, 1975). In the primary differentiation of the accessory glands in the pupa, pharate adult and post-ecdysial adult, the anlagen go through cell divisions, morphogenetic movement, and then proceed to manufacture differentiation-specific proteins. Certain of the differentiative transitions must be autonomous and others dependent upon hormonal signals from the corpora allata or other endocrine centers. The role of hormones in the primary differentiation of accessory glands of holometabolous insects has been little investigated. We intend to evalute the parts played by endocrine signals in the primary differentiation of the SAG on the basis of both biochemical and morphological indices.

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