THE JOURNAL OF EXPERIMENTAL ZOOLOGY 220:81-92 (1982)

Cytodifferentiation in the Accessory Glands of *Tenebrio molitor*. VII. Patterns of Leucine Incorporation by the Bean-Shaped Glands of Males

GEORGE M. HAPP, CHRISTINE YUNCKER, AND PATRICK J. DAILEY
Department of Zoology, University of Vermont, Burlington, Vermont 05405

ABSTRACT The bean-shaped accessory glands of male mealworm beetles are morphologically complex. Seven distinct cell types produce a semisolid secretory mass that contains structural proteins of the spermatophore. Cell numbers increase 3.5-fold over the pupal instar, and gland volume increases 30-fold over the pupal and early adult stage. DNA content reaches its maximum at adult ecdysis (11 pg/secretory cell), RNA at 4 days later (55 pg/cell), and protein at 6 days after ecdysis (1,200 pg/cell). Rates of ^{14}C- and ^{3}H-leucine incorporation increase in parallel to the rise in protein content. Over ten putative secretory protein bands were identified by using one-dimensional SDS-slab gel electrophoresis to compare BAG homogenates with homogenates of the secretory mass and the spermatophore. At least five of these secretory proteins accumulate in BAGs after adult ecdysis, and most show high rates of leucine incorporation in the adult. Two-dimensional gel electrophoresis (pI and SDS) and fluorography allowed us to identify a score of reference spots, at least seven spots that are characteristic of the pupa, and over 40 spots that appear to be differentiation-specific and are presumably secretory products. The fluorographic indices give an unambiguous means of scoring terminal differentiation in the BAG.

In insects, there is little differentiation of accessory sex glands until the onset of adult development in the penultimate instar. The glands grow rapidly, usually after a fall in the titer of juvenile hormone. Terminal differentiation culminates in a massive export of secretory products. Depending on the reproductive biology of each species, the secretory episode may be brief or sustained over a long period. We are interested in peak differentiation and its hormonal modulation in the accessory glands of the yellow mealworm beetle, *Tenebrio molitor*.

Male mealworm beetles possess two pairs of accessory reproductive glands, the bean-shaped glands (BAGs) and the tubular glands (TAGs). Secretory products of the TAGs and the BAGs contribute to the seminal fluids and to the spermatophore or sperm sac (Gadzama, '72; Poels, '73; Gerber, '76; Dailey et al., '80; Black and Happ, '81). The TAGs possess a single morphological type of secretory cell and elaborate five major classes of differentiation-specific proteins, differing from one another in molecular weights and in isoelectric points (Happ et al., '77; Black and Happ, '81; Happ, unpublished). The BAGs are more complex in their morphology. There are seven types of secretory cells, each of which is confined to a particular zone of the gland epithelium. The secretory product of the gland as a whole is a viscous, ordered plug with seven distinct layers. Each layer can be related to a particular cell of origin. The bilaterally symmetrical plugs ooze out of the lumens of the two BAGs into the median ejaculatory duct where they fuse (Dailey et al., '80). Histochemical and immunochemical evidence indicates that the BAG products form part of the spermatophore (Gadzama, '72; Frenk and Happ, '76). Little is known of the control of differentiation in this gland. The present paper chronicles some aspects of the changing biochemical phenotypes as BAG develops over the pupal and adult stages, and provides a basis for scoring the extent of terminal differentiation.

Patrick J. Dailey's present address is University of Southern Illinois, School of Dentistry, Alton, IL 62002.

0022-104X/82/2201-0081$03.50

MATERIALS AND METHODS

Insect cultures

Mealworms were obtained from commercial sources and also from laboratory colonies at the University of Vermont. Freshly eclosed pupae were collected, sexed, and maintained at 26°C until used for experiments. Synchronously eclosed adults were similarly collected and maintained in groups of ten or less. Colonies were fed Purina Chick Startena and potato or carrot.

Gland volumes

Volumes of glands were determined from the weights of plasticene scale models. Pupae or beetles were dissected in saline, the glands removed, and their various aspects projected with the camera lucida. A plasticene model was trimmed and shaped until it conformed well to the outlines of the gland, and the model was checked by comparison with projections of three to five additional glands. The models were weighed and the volumes of the actual BAGs calculated by adjustment to scale.

Cell numbers

Cell numbers were estimated by an adaptation of the techniques of Selman and Kafatos ('75). Glands were washed in saline, minced, and transferred to 2 ml of 0.1 M Tris HCl (pH 7.48) and homogenized with a Dounce tissue grinder (seven strokes with loose-fitting pestle A followed by two strokes with tight-fitting pestle B). Nonidet NP-40 was added to a concentration of 0.5% and the homogenate subjected to two strokes of pestle B. Azure blue (0.01%) was added to stain the nuclei and the homogenate spun at 2,000g for 7 minutes. The resulting pellet was resuspended and aliquots counted in a hemocytometer.

Protein, RNA, and DNA content

Glands were dissected in saline and homogenized in distilled water, and the protein content was determined by the Lowry procedure (Lowry et al., '52) using bovine serum albumin as the standard. RNA content was determined in similar samples after TCA precipitation of the protein, base hydrolysis, and reading of absorption at 260 nm (Happ et al., '77). DNA content was determined by the diphenylamine procedure using calf thymus DNA as the standard (Burton, '69).

Leucine incorporation

Tritium-labeled leucine (0.5 μl of 3,4,5 ^{3}H; specific activity 60 C/mM) was injected into the hemocoel to allow the leucine to be incorporated into TCA-precipitable proteins. A preliminary series of experiments with 6-day adults showed that leucine incorporation increased rapidly over the first 2 hours and slightly between 2 and 4 hours, and thereafter no increase in radioactivity of precipitable proteins was detectable. We injected tritiated leucine (0.5 μC) into pupae or adults and routinely allowed 4 hours for incorporation in vivo. The experiments were terminated by chilling the beetles. Glands were removed and homogenized, and the protein was precipitated and washed via the procedures of Kennell ('67). All TCA washes contained 10 mM cold leucine.

Application of Bartlett's test to the raw data indicated that the values for subsequent days were heteroskedastic (variance significantly heterogenous). A log transformation was performed to obtain homogeneity of variance before the 95% confidence intervals were calculated (Sokal and Rohlf, '69). Any datum point that differed by more than 3 standard deviations from the daily mean was omitted from the final calculations; this procedure eliminated no more than one datum point fore each day.

Electrophoresis and fluorography

Samples for electrophoresis (60 μg of protein in a 30 μl volume) were prepared in Ames sample buffer (Ames and Nikaido, '76). Electrophoresis of proteins on isoelectric (pI) polyacrylamide tube gels and on 12% sodium dodecyl sulphate (SDS) polyacrylamide slab gels followed the technique of O'Farrell ('75) with the omission of the prerun. Gels were stained with Coomassie Blue. Either tritiated or ^{14}C-labeled leucine (U-labeled, 300 mC/mM) was injected for fluorography or autoradiography. Radioactive protein standards (New England Nuclear) were used to determine the molecular weights of the major BAG proteins.

For fluorography, slab gels of radioactive proteins were impregnated with diphenyloxazole in dimethyl sulfoxide, dried, and pressed against Kodak X-Omat film for exposure at −70°C (1–180 days) (Bonner and Laskey, '74). To quantify the relative leucine incorporation into major bands from homogenates of mature BAGs, the bands were excised from a slab gel that had been used for fluorography after solubilizing with Protosol (0.4 ml, 2 h, 50°C), and the radioactivity was determined with a Beckman LS-7000 scintillation counter.

To confirm the identity of actin in our homogenates, soluble proteins from BAG in 0.5 M sodium acetate, 7 mM $CaCl_2$, 30% glycerol, were poured through a DNAse I-agarose affini-

ty column (Worthington) and the adhering material eluted stepwise with 0.75 M guanidine HCl and 3.0 M guanidine HCl in the above buffer. Authentic actin (Millipore Corp.) was similarly processed (Lazarides and Lindberg, '74).

RESULTS

At pupal ecdysis, each BAG/TAG pair looks something like a pair of mittens, lying side by side with the thumbs inward. In the pupal stage and through the first week after adult ecdysis, the glands grow in size. As described above, we made scale models of the BAGs at 2-day intervals over this period of rapid growth. The models are shown in Figure 1. Each was carefully molded to be a "consensus representation" of 3–5 typical glands of that particular age. The volume of each model was determined by weighing it, and the volume of the gland was calculated. The growth in volume fits a simple exponential model (Fig. 2).

The increases in protein, RNA, and DNA content are shown for the whole gland in Figure 3. DNA content reaches its maximum at adult ecdysis; RNA continues to accumulate for 4 days longer, and protein content increases until 7–8 days after ecdysis. The total leucine incorporation increases steadily over the pupal and early adult period (Fig. 4).

Mitoses in the pupa (Grimes and Happ, '80) account for the fourfold increase in cell numbers, from about 5×10^4 cells/gland at pupal ecdysis to about 2×10^5 cells/gland at adult ecdysis. Changes in DNA content parallel those in cell numbers. Our data indicate that there is about 11 pg of DNA per cell at all ages. RNA and protein increase on a per cell basis as shown in Table 1.

One-dimensional slab gels, stained with Coomassie Blue, revealed over 40 protein bands in homogenates of mature BAGs (Fig. 5). In an attempt to determine which bands represent secretory proteins we compared those from the BAG with those from other samples enriched in its products: the semisolid secretory plug and the spermatophore. Secretory plugs were teased out of the BAG and also from the ejaculatory duct by dissection. Spermatophores were collected by interrupting copulation 30 seconds after it had begun, when the elongate spermatophore was protruding from the aedeagus. All samples were run in parallel. On Figure 5, small arrows on the right indicate the bands that are present in all samples. We believe these bands are among the export proteins of the BAG. Their approximate molecular weights are indicated on the right. The prominent band at 43,000 daltons (43 K) is absent from the spermatophore, and therefore appears to be an intracellular protein that had contaminated the dissected secretory plugs. We suspected that this band was actin. To confirm this suspicion, we poured a BAG homogenate through a DNAse-agarose column and eluted with guanidine-HCl, as described above. The 43 K protein was preferentially bound to DNAse-agarose and eluted with guanidine, as was authentic actin. Apparently the actin remains in the cells and does not form part of the spermatophore.

When homogenates of BAGs of increasing ages were compared on a single gel, the pattern of banding shows marked changes with age (Fig. 6). Of the putative export proteins identified earlier, those with molecular weights of 78 K, 46.6 K, and 24.8 K are very lightly stained at adult ecdysis and most heavily stained in glands of mature adults (6 days or more after ecdysis).

Fluorography after one-dimensional SDS gel electrophoresis shows high rates of leucine incorporation into those bands already assumed to be export proteins and also into several others which are hard to distinguish on the Coomassie-stained gels (Fig. 7f). The incorporation pattern was quite reproducible, including very marked leucine incorporation in high molecular weight bands (84 K and 78 K) of glands from mature adults. The relative rates of leucine incorporation into the major bands were determined more exactly by cutting out the bands and solubilizing them with protosol. Table 2 summarizes the incorporation for the major bands of glands from 8-day adults. One-half of the total incorporation is restricted to two bands of high molecular weight, and almost another third is in bands of 20,000 daltons or less. In some other insect systems, such as the fat body of *Locusta*, which is responsible for the synthesis of vitellogenins a large translation product is cleaved into significantly smaller secretory products (Chen et al., '78; Chen, '80). We attempted to detect a similar cleavage processing in the BAG by comparing incorporation patterns among several time periods, varying from 5 minutes to 24 hours. No differences were seen among the various incorporation intervals (data not shown), but additional studies will be required before we can rule out posttranslational processing in our system.

A clear shift in the relative rates of leucine incorporation takes place as the BAGs mature (Fig. 7). Just after pupal ecdysis (Fig. 7a), there

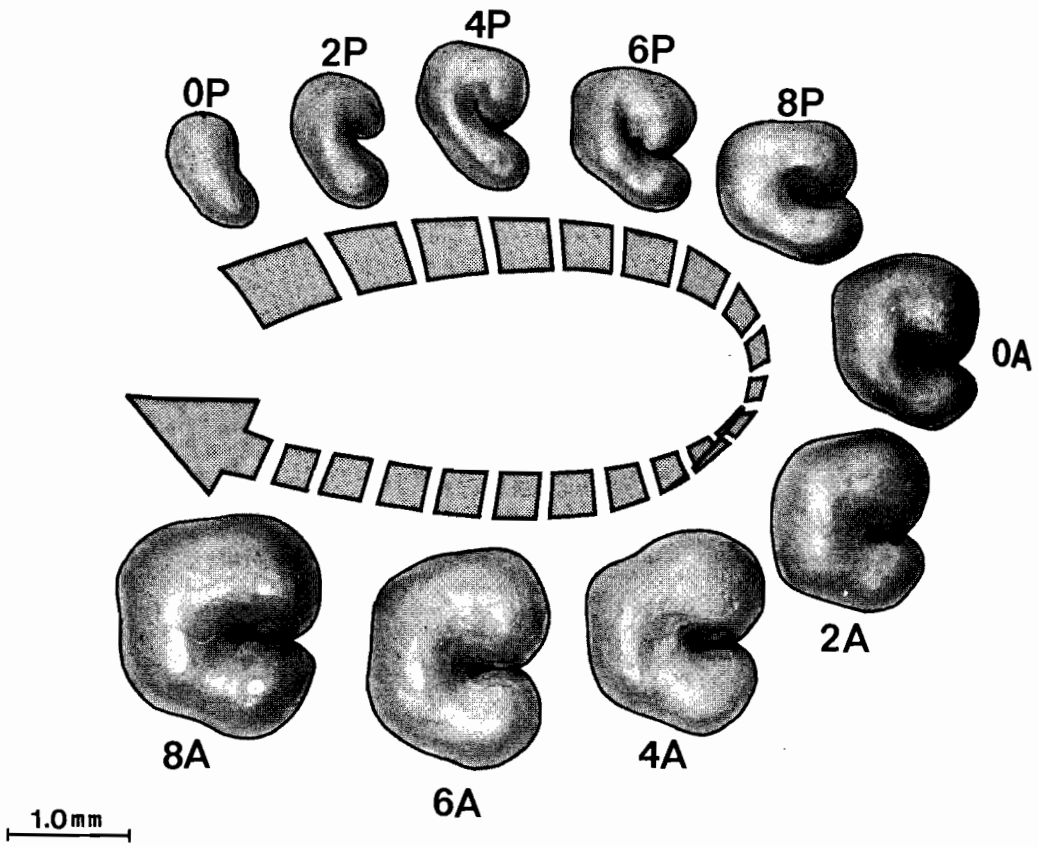


Fig. 1. Scale models of the BAG during the 9-day pupal stage (days 0, 2, 4, 6, and 8 are shown) and over the first 8 days of the adult instar (days 0, 2, 4, 6, and 8 are shown).

is much leucine incorporated into proteins of 68 K, 60 K, 53 K, 50 K, and 43 K daltons. In the midpupal stage (3–6 days later), leucine is increasingly incorporated into proteins of 78 K, 72 K, 37.2 K, 35 K, 33 K, 18 K, and 16 K daltons. Just after adult ecdysis, proteins of intermediate weights (70 K–43 K daltons) show the most incorporation. Incorporation into a protein at 84 K is just beginning, and over the next 6 days the bands at 78 K and 84 K come to predominate. In the postecdysial adult maturation, bands at 47.6 K, 24.8 K, and several between 21 K and 14 K show increasing incorporation, whereas the presumed actin progressively drops in its rates of incorporation.

In summary, both the "accumulation profile" of proteins stained with Coomassie Blue and the incorporation profile from the fluorographs show a marked emphasis on molecular weights which resemble the secretory proteins.

The one-dimensional gels left several ambiguities, which may stem from the fact that several proteins may be indistinguishable in molecular weight. For example, both midpupae and mature adults show high incorporation into a band at 78 K and a cluster of bands below 20 K: Do these similarities reflect a recurrent synthesis of the same proteins? Do the consistent leucine incorporations at 46 K to about 60 K indicate the synthesis of the same proteins throughout development? Many of the ambiguities were resolved by separating the proteins on two-dimensional pI-SDS gels.

The two-dimensional gels demonstrate that pupae and adults have very different patterns of leucine incorporation (Fig. 8). Some protein spots are common to all developmental stages, and these reference spots have been indicated as the fluorographs with letters A to P (Fig. 8). Of particular utility in comparing different

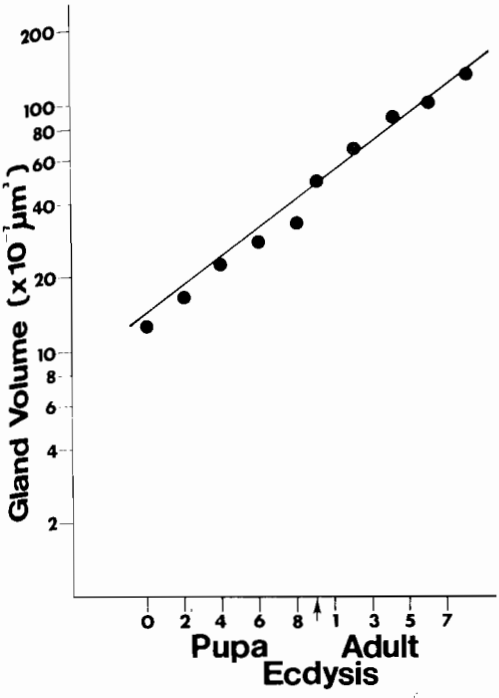


Fig. 2. Increase in volume of the BAG during development from 0-day pupa to 8-day adult. The regression equation for the line shown is log y = 0.059x + 1.12, r = 0.9982.

gels are two clusters of spots that form "constellations," which are joined in the figure by dashed lines.

Constellation A–G varies in molecular weight from 43 K daltons (A, which is actin) to 69.5 K daltons (E) and in isoelectric point from 5.25 (C) to 5.8 (G). This constellation includes many of the bands seen on the one-dimensional gels (Fig. 7). The two-dimensional gels also show that the actin spot is a chain of three, varying slightly in isoelectric point. We assume that the predominant actin corresponds to actin II of *Drosophila* and that the more basic variant at midpupa is muscle actin (see Horovitch et al., '79; Fryberg et al., '80). Constellation H–M varies in molecular weight from 30.5 K daltons (M) to 40 K daltons (H) and in pI from 5.0 (I) to 5.1 (J).

Both pupal and adult stages have characteristic sets of protein spots in the BAG homogenates. The most prominent pupal spot is 1, at 78 K daltons with a pI of 5.4–5.8. It is prominent only in the midpupa and sometimes is visible as a minor spot in freshly ecdysed adults. Spot 1 was not visible in the homoge-

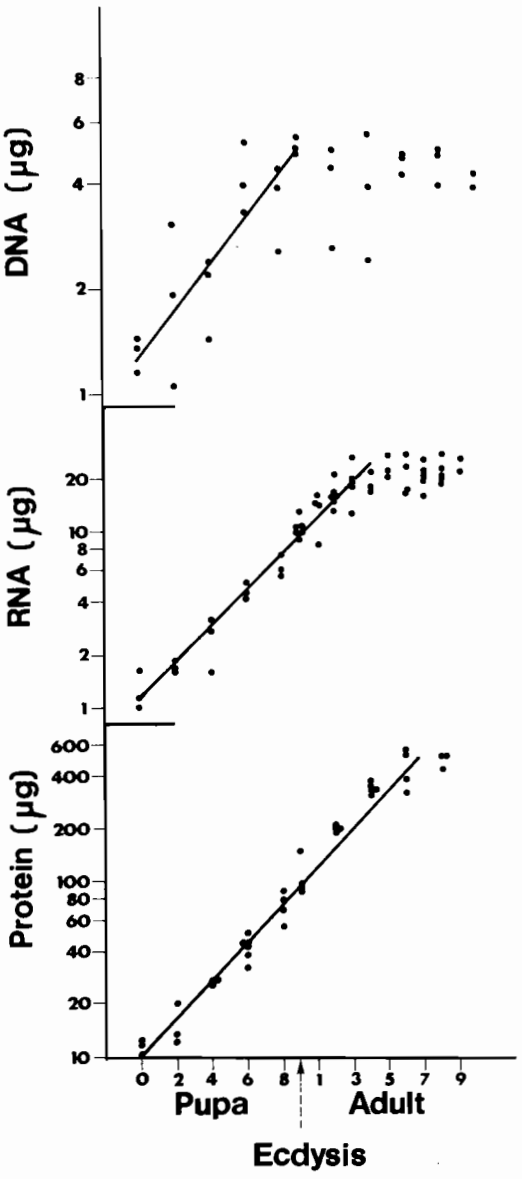


Fig. 3. DNA, RNA, and protein content per pair of BAGs over the pupa and early adult stage. All data points are shown. Determinations as described in Materials and Methods. The regression lines shown were calculated as follows: DNA over pupal days 0–9; log y = 0.0685x + 0.0936, r = 0.8337. RNA over the pupal stage and through adult day 4: log y = 0.101x + 0.0644, r = 0.9729. Protein over pupal stage and through adult day 6: log y = 0.110x + 0.966, r = 0.982.

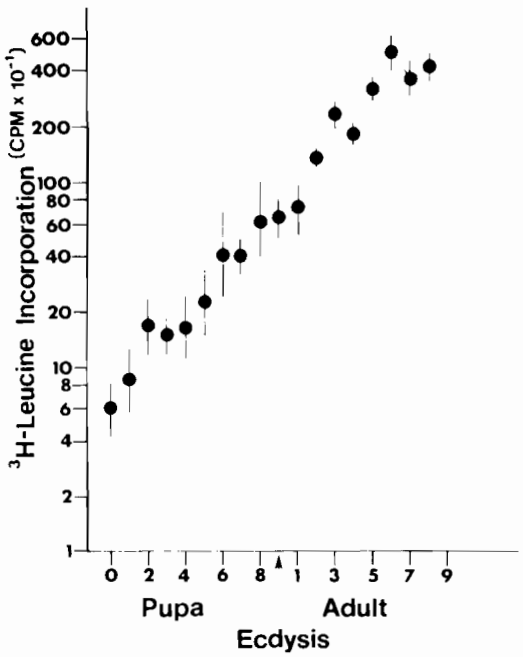


Fig. 4. ^{3}H-leucine incorporation into TCA-precipitable proteins of BAG. Labeled leucine was injected and protein was isolated as described in Materials and Methods. A minimum of eight gland pairs was examined at each day. The mean value and 95% confidence intervals are indicated.

TABLE 1. RNA and protein per cell of BAG

	0-day pupa	Adult ecdysis	8-day adult
RNA/cell	9.4 pg	23 pg	55 pg
Protein/cell	77 pg	250 pg	1230 pg

nate shown at 7c. Spot 3, a basic protein of about 16 K daltons, is present throughout the pupal stage, but leucine incorporation falls off sharply at adult ecdysis (Fig. 8c).

There are a considerable number of adult-specific spots, some of which are obvious after a short exposure (as seen in Fig. 8d or f) whereas others are quite dark only after a longer exposure of the X-ray film (Fig. 8e). The heaviest incorporation is seen in spot 58, a smear from about 76 K daltons to about 90 K daltons and with pIs that range between 5.0 and 5.4. We first see this spot at ecdysis (Fig. 8c). This spot presumably represents at least the two bands (at 78 K and 84 K) seen on one-dimensional fluorographs, which accounted for about half of the leucine incorporation in a mature gland. Spots 10, 11, and 12 are next most prominent. They are clustered at low molecular weights (14.4–18 K daltons) and at a pI of 5.7–5.8 (Fig. 8c–f). Spots 24 (24.8 K daltons), 56 (46.6 K daltons), 55 (50 K daltons), and 62 (high molecular weight) also show quite high levels of leucine incorporation. Longer exposures reveal a prominent cluster of spots, 15–23, which vary in molecular weight from 22.6 to 27.3 K daltons and a pair of distinct spots at 46.6 K daltons (53 and 54). The last mentioned pair probably coincides with spot H (47.2 K daltons) to form the band at 47 K daltons on one-dimensional fluorographs of mature BAG (Fig. 7f). Although a variety of other spots are adult specific, the ones discussed suffice for indices of terminal differentiation.

DISCUSSION

Over the 9 days of the pupal stage and the first 5 days of the adult, the BAG increases steadily in size and in protein content. Two lines of evidence suggest that almost all of the protein synthesis is endogenous, i.e., little preformed protein is absorbed from the hemolymph and stored in the BAG. Ultrastructural studies have detected no significant endocytosis at the basal margins of the elongate secretory cells of the BAG (Gadzama, '72; Grimes and Happ, '80; Dailey et al., '80), and when mature BAGs are labeled with leucine in vitro (culture medium), all the major bands show incorporation by a one-dimensional electrophoresis and fluorography (unpublished data). *T. molitor* usually do not mate until 5 days after adult ecdysis, so secretory proteins accumulate until that time. Thus, the rate of protein accumulation is itself a reasonable minimum estimate of the rate of protein synthesis.

In the present study, we injected a constant amount of ^{3}H-leucine (0.5 μC) into all ages, and the rate of tritium incorporation rose in parallel to the rate of protein accumulation. With each daily logarithmic incremental increase, there is a proportionate increase in leucine incorporation. Thus, the specific activity of the synthesized protein is approximately constant, and we believe it probable that the specific activity of the leucine precursor pool is constant. The absolute size of the *intra*cellular leucine pool is quite likely to be increasing over the postecdysial period of rapid growth in cell volume, but the effective pool size for our labeling conditions apparently does not change. This constancy can be explained by rapid equilibration of leucine pools in the hemolymph and in the secretory cells of the BAG. The rapid movement of leucine into the cellular pools is reflected in the fact that a "nor-

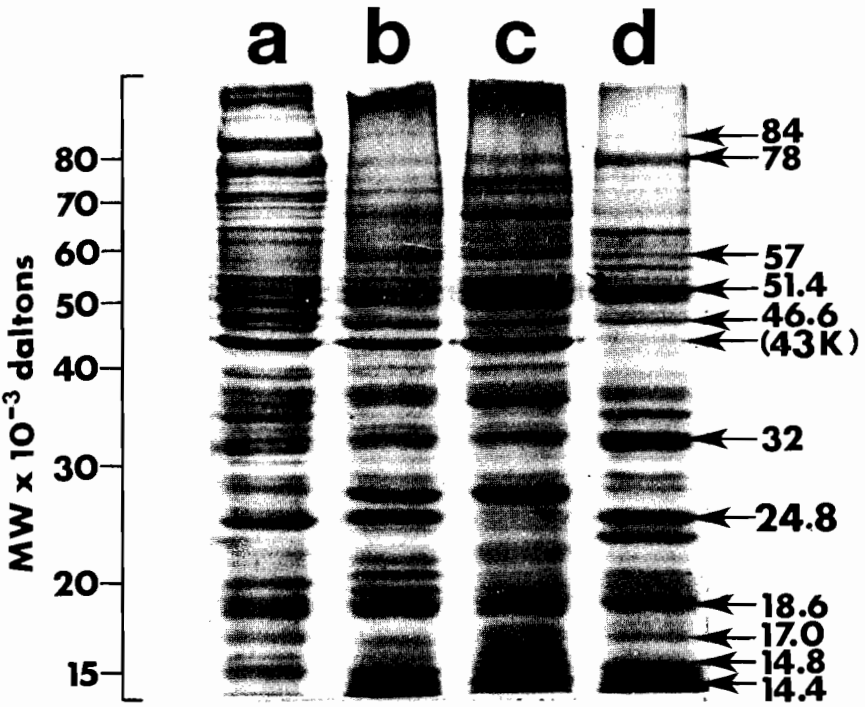


Fig. 5. Polyacrylamide gel electrophoresis of BAG homogenate and its secretory products on a 12% SDS slab. a) BAG of eight day male, b) secretory plug from BAG lumen, c) secretory mass from ejaculatory duct, d) spermatophore. Molecular weights on the right in kilodaltons. Coomassie Blue stain.

mal" labeling pattern is seen in the fluorographs after only 5 minutes of incorporation. The lack of significant exogenous synthesis of secretory proteins and the effective constant size of the leucine precursor pool make it convenient to use leucine incorporations as accurate indices of the rates of protein synthesis in this system.

The rapid growth in volume of the BAG appears to be well described by an exponential model (Fig. 2), but that superficial simplicity is misleading. Careful examination of the quantitative data on gland volumes (Fig. 2) shows a slight inflection in the data points, which coincides with adult ecdysis. "Slackening of the rate of growth" is often reported at "special epochs in a lifetime" (p. 161, Thompson, '42). The inflection coincides with a shift from mitoses to cell growth. Such a growth pattern, first mostly in cell number and later exclusively in cell size, is commonly observed in other insect systems, such as the wings of certain *Drosophila* (Dobzhansky, '29; Goldschmidt, '37).

Once the BAGs have reached their fully functional state, the beetles continue their reproductive activity for several months. We do not know whether the BAGs continue to secrete plugs at a constant rate over that period, or whether biosynthetic activity wanes between sexual encounters. The spermatophore is produced within 1 minute of the start of copulation (Gadzama and Happ, '74), and this fact suggests that at least one set of precursors is stored "in anticipation" at all times.

In many animals with long-lived adult stages, there are cycles of reproductive activity and recurrent episodes of secretion production in the accessory sex glands. Once the primary stimulation by sex steroids has induced the differentiation of the oviduct of a chicken (Palmiter, '75) or of the prostate of a rat (Parker et al., '78), the recurrent secretory pattern follows rapidly from a second rise in circulating hormones (in these cases, estrogen and testosterone, respectively). The recurrent secretion from secondary stimulation involves increased production of export proteins. The initial increase is not dependent upon cell proliferation, although mitosis takes place before

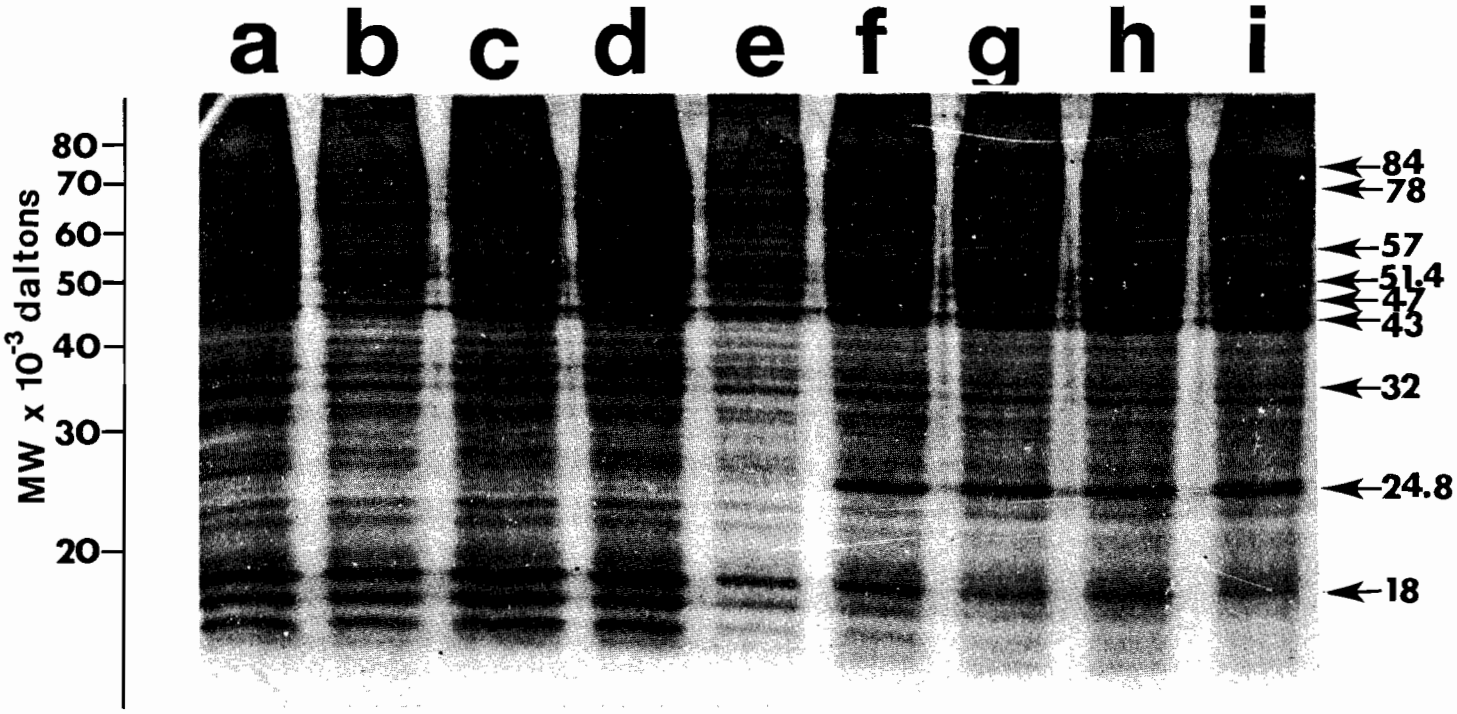


Fig. 6. Polyacrylamide gel electrophoresis of BAG homogenates on a 12% SDS slab. The ages of the beetles are as follows: a) 0-day pupa, b) 3-day pupa, c) 6-day pupa, d) 9-day pupa, e) 0-day adult, f) 2-day adult, g) 4-day adult, h) 6-day adult, i) 8-day adult. The arrows on the right indicate several of the adult-specific proteins with their molecular weights in kilodaltons. Coomassie Blue stained.

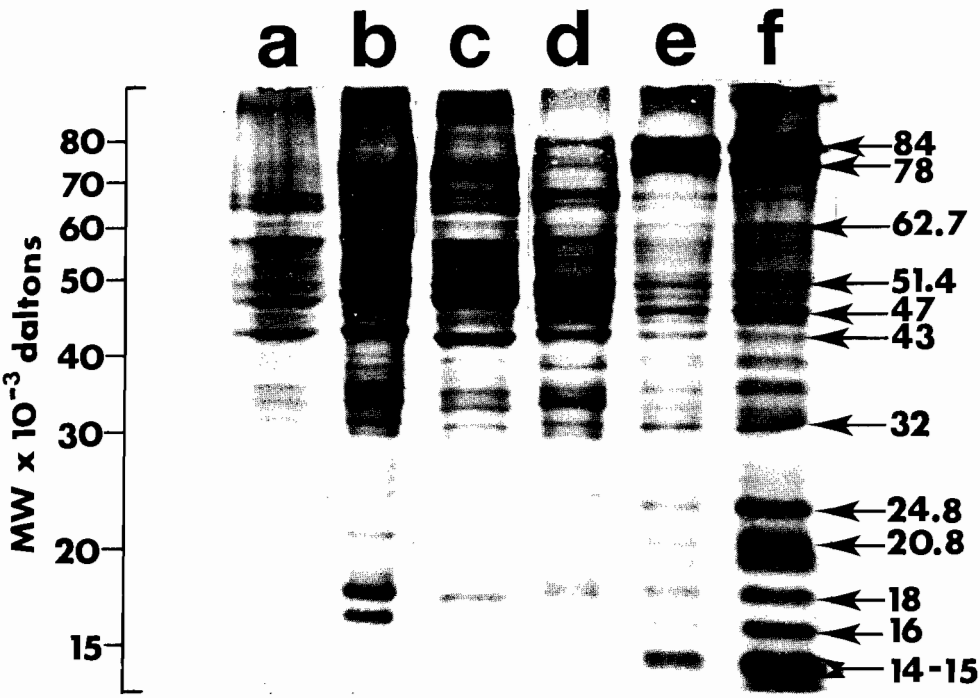


Fig. 7. Fluorograph of BAG homogenates after one-dimensional electrophoresis of ^{14}C-leucine-labeled samples: a) 0-day pupa, b) 3-day pupa, c) 6-day pupa, d) 0 day-adult, e) 3-day adult, f) 6-day adult. Molecular weights on the right in kilodaltons.

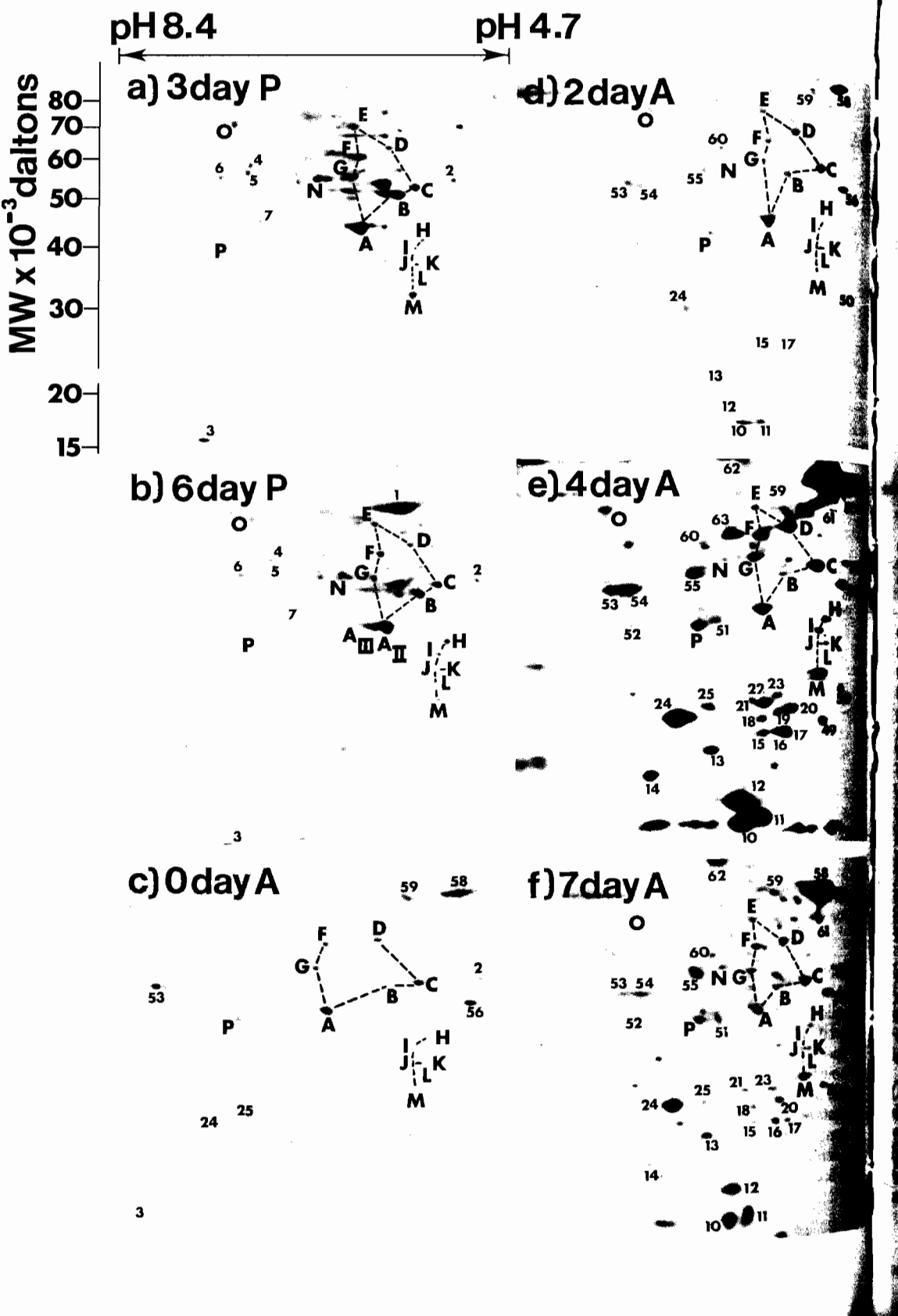
*TABLE 2. Incorporation of ^{14}C-leucine into bands of SDS-gel**

Molecular weight	% Recovered
84 K	36 ± 7
78 K	15 ± 2.6
47 K	6.5 ± 1.6
43 K	3.6 ± 0.9
32 K	3.7 ± 0.8
24.8 K	6.1 ± 1.6
20.8 K	
18 K	
16 K	29.3 ± 4.5
14 K	

*Averages are of three experiments with over 3,000 CPM applied to each lane.

the maximum production is achieved (Palmiter et al., '76; Parker et al., '78). Some insect glands follow a similar pattern of recurrent synthetic activity. The milk glands of tsetse flies are special female accessory glands that provide nourishment for the internally developing larva. With the start of each pregnancy of the tsetse, there is an increase in cell size, in content of rough endoplasmic reticulum, and in secretion production (Bonnanfant-Jaïs, '74, '75; Denlinger and Ma, '74; Ma et al., '75). Within a few hours of parturition the cells involute, and with a successive pregnancy a recurrent secretory cycle follows. No cell division takes place during these recurrent cycles. Ablation of the medial neurosecretory cells of the brain (Foster, '74) or removal of the corpora allata (Ejezie and Davey, '76) interferes with fecundity, perhaps by directly affecting the milk gland, but relatively little is known about the endocrine control of milk gland secretions.

We know something of the fluctuations of hormones during accessory gland development in *T. molitor*. The ecdysteroid levels rise and fall in midlarval instar (Delbecque et al., '78; Lack and Happ, '76). In prepupae, there may be ecdysteroid peaks at earlier times, but the major peak occurs about three-quarters of the way through the instar (Delbecque, '75). The major pupal peak of ecdysteroids is at ocular stage 4-6, approximately at mid-instar (Delbecque et al., '80). In the BAG and the TAG, this peak coincides with the midinstar synthesis of 78 K dalton proteins and the maximum synthesis of the protein of spot 3 (16 K daltons). In adult female *T. molitor*, and perhaps in males as well, the corpora allata


pH 8.4
pH 4.7
MW x 10⁻³ daltons
80
70
60
50
40
30
20
15
a) 3day P
b) 6day P
c) 0day A
d) 2day A
e) 4day A
f) 7day A

begin to produce juvenile hormone as early as 48 hours after ecdysis (Weaver et al., '80). Juvenile hormone could trigger the terminal differentiation. The importance of the pupal ecydsteroid peak and the adult juvenile hormone surge to BAG development is now under study in our laboratory.

The coordinate differentiation of the cells of the pupal BAG is probably affected not only by developmental hormones, but also by the intriguing intercellular bridges present in the pupal stage (Grimes and Happ, '80). We do not yet know whether all seven cell types mature in tight synchrony. We do know they have completed their development by the first copulation at 5 days after ecdysis. Patterns of leucine incorporation reported in this paper suggest some asynchrony in the achievement of peak incorporation into the export proteins. In Figure 8c and d (above), the 78 K and 84 K spots (58) show a marked increase in leucine incorporation at 0 and 2 adult days, before the maximum of many other export proteins. Asynchrony in reaching peak incorporation may reflect the fact that each secretory cell type matures according to its own schedule, or it may stem from progressive changes within a single cell type that produces several proteins.

Each of the seven cell types of BAG produces its own morphologically unique secretory granules that pass into a distinct layer of the plug (Dailey et al., '80). We are not yet able to correlate a band on the fluorographs with a granule from one of the seven secretory cell types. The structural plug is produced by cells that secrete in parallel with one another, and we suggest that there must be a coordinated microsequence of secretory episodes such that each cell population "meters out" an appropriate quantity of product at the appropriate times to form the sequential layers. In order to understand better both development and secretory coordination and synchrony, we must map the bands on the fluorographs to the various cell types.

The changes in leucine incorporation that take place as the BAG matures offer precise and unambiguous indices of the stage of differentiation. We aim to use indices to ask which developmental transitions are hormone-dependent and which are autonomous.

Fig. 8. Fluorographs of BAG homogenates after two-dimensional electrophoresis, pI tube gel in the horizontal and SDS slab gel in the vertical direction. Ages of the pupae (P) and adults (A) are shown on the photographs. Small A-P represent reference spots, characteristic of all ages. Numbered spots are typical of pupal (1-7) or adult (> 10) stages.

ACKNOWLEDGMENTS

We thank Bonnie MacLeod for the DNA content measurements, Margaret Grimes for the nuclear counts, the National Institutes of Health (AI-15662 and GM-26140) for financial support, Tom Wilson and Paul Black for their critical comments on an earlier draft of this manuscript, and Joyce Murray for her patient retyping of the various drafts.

LITERATURE CITED

Ames, G.F.-L., and K. Nikaido (1976) Two-dimensional electrophoresis of membrane proteins. Biochemistry, *15*: 616-623.

Black, P.N., and G.M. Happ (1981) Cytodifferentiation in the accessory glands of *Tenebrio molitor*. VIII. Crossed-line immunoelectrophoretic analysis of terminal differentiation in the post-ecdysial tubular accessory glands. (Submitted).

Bonnanfant-Jaïs, M.L. (1974) Morphologie de la glande lactée d'une glossine, *Glossina austeni* Newst, au cours du cycle de gestation. I. Aspects ultrastructuraux en période de gestation. J. Microsc. (Paris), *19*:265-284.

Bonnanfant-Jaïs, M.L. (1975) Morphologie de la glande lactée d'une glossine, *Glossina austeni* Newst, au cours du cycle de gestation. II. Aspects ultrastructuraux en période de repos et au cours de gestations successives. J. Microsc. (Paris), *24*:295-314.

Bonner, W.M., and R.A. Laskey (1974) A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem., *46*:83-88.

Burton, K. (1968) Determination of DNA concentration with diphenylamine. In: Methods in Enzymology XIIB. L. Grossman and K. Moldave, eds., pp. 163-166.

Chen, T.T. (1980) Vitellogenin in locusts (*Locusta migratoria*): Translation of vitellogenin mRNA in *Xenopus* oocytes and analysis of the polypeptide products. Archiv. Biochem. Biophys., *201*:266-276.

Chen, T.T., P.W. Strahlendorf, and G.R. Wyatt (1978) Vitellin and vitellogenin from locusts (*Locusta migratoria*). Properties and posttranslational modifications in the fat body. J. Biol. Chem., *253*:5325-5331.

Dailey, P.J., N.M. Gadzama, and G.M. Happ (1980) Cytodifferentiation in the accessory glands of *Tenebrio molitor*. VI. A congruent map of cells and their secretions in the layered elastic product of the male bean-shaped gland. J. Morphol., *166*:289-322.

Delbecque, J.-P. (1976) Taux d'ecdysones et cycle de mue au cours de la métamorphose de *Tenebrio molitor* L. (Insecte, Coléoptère). Third cycle doctoral thesis, University of Dijon.

Delbecque, J.-P., M. Hirn, J. Delachambre, and M. DeReggi (1978) Cuticular cycle and molting hormone levels during the metamorphosis of *Tenebrio molitor* (Insecta Coleoptera). Dev. Biol., *64*:11-30.

Denlinger, D.L., and W.C. Ma (1974) Dynamics of the pregnancy cycle in tsetse *Glossina morsitans*. J. Insect Physiol., *20*:1015-1026.

Dobzhansky, T. (1929) The influence of quantity and quality of chromosomal material on the size of the cells in *Drosophila melanogaster*. Wilh. Roux Archiv. Entwicklungs., *115*:363-379.

Ejezie, G.C., and K.G. Davey (1976) Some effects of allatectomy in the female tsetse, *Glossina austeni*. J. Insect Physiol., *22*:1743-1749.

Foster, W.A. (1974) Surgical inhibition of ovulation and gestation in the tsetse fly *Glossina austensi*. Newst. Bull. Entomol. Res., *63*:483-493.

Frenk, E., and G.M. Happ (1976) Spermatophore of the mealworm beetle: Immunochemical characteristics suggest affinities with male accessory gland. J. Insect Physiol., *22*:891-895.

Fryberg, E.A., K.L. Kindle, N. Davidson, and A. Sodja (1980) The actin genes of *Drosophila*: A dispersed multigene family. Cell. *19*:365-378.

Gadzama, N.M. (1972) A histological, histochemical, and electron microscopical study of the spermatophore and the internal reproductive organs of male *Tenebrio molitor* L. (Coleoptera:Tenebrionidae). Ph.D. Thesis, New York University.

Gerber, G.H. (1976) Reproductive behaviour and physiology of *Tenebrio molitor* (Coleoptera:Tenebrionidae). III. Histogenetic changes in the internal genitalia, meserteron, and cuticle during sexual maturation. Can. J. Zool., *54*: 990-1002.

Goldschmidt, R. (1937) Gene and character. IV. Further data on the development of wing mutants in *Drosophila*. Univ. Calif. Pubs. Zool., *41*:277-282.

Grimes, M.J., and G.M. Happ (1980) Fine structure of the bean-shaped gland in the male pupa of *Tenebrio molitor* L. (Coleoptera:Tenebrionidae). Int. J. Insect Morphol. Embryol., *9*:281-296.

Happ, G.M., C. Yuncker, and S.A. Huffmire (1977) Cytodifferentiation in the accessory glands of *Tenebrio molitor*. II. Patterns of leucine incorporation in the tubular glands of post-ecdysial adult males. J. Exp. Zool., *200*:223-236.

Horovitch, S.J., R.V. Storti, A. Rich, and M.L. Pardue (1979) Multiple actins in *Drosophila melanogaster*. J. Cell Biol., *82*:86-92.

Kennell, D. (1967) Use of filters to separate radioactivity in RNA, DNA, and proteins. Meth. Enzymol., *12*:686-693.

Lack, R.J., and G.M. Happ (1976) Neuroendocrine control of moulting cycle in mealworms: Bioassay of moulting hormone. J. Insect Physiol., *22*:1123-1125.

Lazarides, E., and U. Lindberg (1974) Actin is the naturally occurring inhibitor of deoxyribonuclease. I. Proc. Natl. Acad. Sci. USA, *71*:4742-4746.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, ad R.J. Randall (1951) Protein measurement with Folin phenol reagent. J. Biol. Chem., *193*:265-275.

Ma, W.-C., D.L. Denlinger, V. Järlfors, and D.S. Smith (1975) Structural modulations in the tsetse fly milk gland during a pregnancy cycle. Tissue Cell, *7*:319-330.

O'Farrell, P.H. (1975) High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem., *250*:4007-4021.

Palmiter, R.D. (1975) Quantitation of parameters that determine the rate of ovalbumin synthesis. Cell, *4*:189-197.

Palmiter, R.D., P.B. Moore, E.R. Mulvihill, and S. Emtage (1976) A significant lag in the induction of ovalbumin messenger RNA by steroid hormones: A receptor translocation hypothesis. Cell, *8*:557-572.

Parker, M.G., G.T. Scrace, and W.I.P. Mainwaring (1978) Testosterone regulates the synthesis of major proteins in rat ventral prostate. Biochem. J., *170*:115-121.

Poels, A. (1972) Histophysiologie des voies génitales mâles de *Tenebrio molitor* L. (Coléoptère:Tenebrionidae). Ann. Soc. R. Zool., Belg., *102*:199-234.

Selman, K., and F.C. Kafatos (1974) Transdifferentiation in the labial gland of silk moths: Is DNA required for cellular metamorphosis? Cell Diff., *3*:81-94.

Sokal, R.R., and F.J. Rohlf (1969) Biometry. The Principles and Practice of Statistics in Biological Research. W.H. Freeman, San Francisco.

Thompson, D.A. (1942) On Growth and Form, Ed. 2, vol. I. Cambridge University Press, Cambridge, England.

Weaver, R.J., G.E. Pratt, A.F. Hamnett, and R.C. Jennings (1980) The influence of incubation conditions on the rates of juvenile hormone biosynthesis by corpora allata isolated from adult females of the beetle *Tenebrio molitor*. Insect Biochem., *10*:245-254.