in *The Functional Organization of the Compound Eye*, C. G. Bernhard, Ed. (Pergamon Press, New York, 1966), pp. 105–124; for insects see T. H. Goldsmith in *The Physiology of Insecta*, M. Rockstein, Ed. (Academic Press, New York, 1964), vol. 1, pp. 397–462. The fine structure of the rhabdoms of *Palaemonetes* has been studied by E. Eguchi and T. H. Waterman and we thank them for making available to us their unpublished electron micrographs.

3. The fly *Calliphora* has been examined by H. Langer and B. Thorell, *Expl. Cell Res.* **41**, 673 (1965). There are two kinds of rhabdomeres in each retinula; two cells have a pigment with λ_{max} at 470 nm; the other six, at 510 nm. The dipteran retinula, however, is unique in having morphologically separate rhabdomeres.
4. The saline contained in final concentration the following: 524 m*M* Na^+; 612 m*M* Cl^-; 13.3 m*M* K^+; 12.4 m*M* Ca^{2+}; 24.8 m*M* Mg^{2+}; and sufficient $NaHCO_3$ to bring the solution to *p*H 7.
5. The design is but slightly modified from that of P. A. Liebman and G. Entine, *J. Opt. Soc. Amer.* **54**, 1451 (1964). The principal differences are that the photomultiplier tube was an EMI 9558QA, the collecting optics as well as the inverted microscope had Zeiss × 32 Ultrafluar objectives, and the programming circuit in the high voltage supply (Kepco, 2500 volts) was used in the feedback control of dynode voltage instead of a 2C53 regulator triode.
6. Usually, to effect a complete bleaching, an additional light exposure must be given after the preparation has sat many minutes in the dark. This suggests that some of the "metarhodopsin" present at the time curve 3 was recorded is converted—whether directly or indirectly is not known—into a photopigment perhaps identical to the pigment bleached by the initial exposure to yellow light.
7. This interpretation of the experiment can be represented as follows:

$$P_{555} \xrightarrow{h\nu} M_{496}$$
$$M_{496} \xrightarrow{h\nu} \text{colorless products}$$
$$P_{496} \xrightarrow{h\nu} \text{colorless products}$$

where P_{555} and P_{496} are the pigments initially present, and M_{496} is "metarhodopsin." On the basis of the evidence in Fig. 1, however, one might also argue that the rhabdom originally contained only one light-sensitive pigment with λ_{max} at about 540 nm. On irradiation, it formed a mixture of two photoproducts with similar absorption spectra, one of which was light-sensitive and bleached during the second, shorter-wavelength exposure. This alternative explanation can be summarized:

$$P_{540} \xrightarrow{h\nu} M_{496}$$
$$P_{540} \xrightarrow{h\nu} M'_{496}$$
$$M_{496} \xrightarrow{h\nu} \text{colorless products}$$
$$M'_{496} \xrightarrow{h\nu} \text{colorless products}$$

where P_{540} is described by the difference spectrum for the total bleach (curve 1 — curve 4). There is a reason for favoring the first alternative at this juncture; Wald [G. Wald, *Nature* **215**, 1131 (1967)] has reported a pair of pigments in digitonin extracts of crayfish eyes which show a striking parallel to P_{555} and P_{496}. The pigment absorbing at longer wavelengths (λ_{max} at 556 nm in *Procambarus* and 562 nm in *Orconectes*) is converted by light into a long-lived intermediate with maximum absorbancy near 515 nm. The second pigment (maximum absorbancy at 525 nm in *Procambarus* and 510 nm in *Orconectes*) bleaches directly to retinal with no discernable intermediates at room temperature. Moreover, in solution the 562 nm pigment is selectively destroyed at *p*H 9, whereas the 510 nm pigment is broken down by 0.06*M* hydroxylamine. Although we have not been able to show corresponding chemical differences when the pigments are incorporated into the structure of the rhabdom (*Orconectes* or *Palaemonetes*), the fact that the differences exist in solution demonstrates the reality of two pigments.
8. H. J. A. Dartnall, *Brit. Med. Bull.* **9**, 24 (1953).
9. Although the width of the slit in the plane of focus was smaller than a band of microvilli, the rhabdom is sufficiently thick that the slit may be significantly out of focus at the upper and lower edges of the rhabdom, and therefore spread into adjacent bands. Even allowing for this complication, on the hypothesis that one of the pigments is missing from alternate bands of microvilli, one can show that if three spots are separated by intervals of one-half band period, the ratio of the pigment in one of the adjacent pairs of spots will be at least 10:1, regardless of the phase relation of the three spots to the banding system.
10. T. H. Waterman, H. F. Fernandez, T. H. Goldsmith, in preparation.
11. This work was supported by PHS grant NB-03333 to Yale University and postdoctoral fellowship NB 22,547 to H.R.F. We thank Dr. P. Liebman for his advice in the construction of the instrument, and N. Mandell for building it. Most of the experiments reported here were performed at the Marine Biological Laboratories, Woods Hole, Massachusetts.
* Present address: Laboratory of Limnology, University of Wisconsin, Madison.

31 May 1968

Cytodifferentiation during Insect Metamorphosis: The Galea of Silkmoths

Abstract. *In the galea of silkmoths undergoing metamorphosis, generalized epidermal cells, which had previously secreted pupal cuticle, transform into highly specialized cells producing a new protein, the enzyme cocoonase. These cells first segregate by mitosis and displacement, then grow rapidly through endomitosis and accumulation of RNA-rich cytoplasm, and finally begin rapid synthesis of cocoonase. Replication of DNA continues in fully differentiated cells synthesizing cocoonase.*

The maxillary galea of certain saturniid moths (*1*) is a particularly favorable organ for the study of development. During the transformation from pupa to adult, highly specialized zymogen cells develop in the galea from the generalized epidermis in a predictable and swift sequence of events. When fully differentiated, these cells rapidly synthesize a characteristic protein, the cocoon-softening proteolytic enzyme cocoonase (*2*). In each galea, 20,000 cells with an aggregate dry weight of about 0.04 mg produce nearly 0.1 mg of enzyme in about 6 days. Cocoonase synthesis starts abruptly at a predictable stage. Glands at all developmental stages can be studied either in vivo or in short-term organ culture. One or more of these favorable characteristics are found in other metazoan cells that have proved useful for developmental studies in recent years (*3*). We describe here the normal course of development in the galea.

Pupae of *Antheraea pernyi* (diapausing first brood from Japan) and *A. polyphemus* (from North Dakota) were placed at 25° ± 0.5°C, after storage at 4°C for several months. Under these conditions, *A. pernyi* develops into an adult moth in 21 days (*A. polyphemus* in 18 days) measured from retraction of the wing epidermis (the first visible manifestation of development; day 0) to emergence of the mature moth (day 20 or day 17, respectively). Development proceeds according to a regular timetable (*4*). Galeae were excised from animals at the desired stage, fixed in 10 percent aqueous acrolein at 0°C overnight (*5*) or in 6 percent glutaraldehyde in half-strength Weevers' saline (*6*), and embedded in glycol methacrylate (*5*). Sections 1 to 1.5 μm thick were used. Autoradiograms were prepared with Kodak liquid emulsion NTB-2. Unless otherwise indicated, observations refer to *A. pernyi*.

In the diapausing pupa the galeae are two hollow, blood-filled, cuticle-covered projections of the epidermis in the head region, roughly conical in shape (*7*). The epidermal cells are arranged in a single squamous layer, separating the cuticle from the blood. The cells are uniform in appearance, each with a flat nucleus and very sparse cytoplasm, only 2 μm thick (Fig. 1a); they seem indistinguishable from epidermal cells elsewhere in the body.

With the onset of adult development, the cells of the galea, like all epidermal cells, retract from the cuticle, leaving behind them the molting gel, a protein-rich material that will eventually digest the endocuticle (*8*). They enlarge and become cuboidal. On days 2 and 3, administration of H^3-thymidine followed by autoradiography reveals DNA synthesis in most of the cells of the lateral half of the galea. Shortly thereafter, on day 4 and early day 5, mitotic figures appear in the same region, and the epithelium grows thicker as the cells proliferate (Fig. 1b).

By day 7, the lateral and medial regions are strikingly different in appearance. Medially, all cells are of the ordinary epidermal type; they are distributed in a single layer and are beginning to synthesize adult cuticle. Laterally (Fig. 1c), three distinct cell types have appeared following mitosis and cell displacement. At the surface, ordinary epidermal cells are synthesizing cuticle, just as in the medial region. In an intermediate layer, duct cells are elaborating cuticular tubules extending from the surface to the more basally located cells of the third type. The latter are destined to synthesize the enzyme cocoonase, and will be called zymogen cells (*9*); they are distinguished by enlarged, presumably polyploid, nuclei and by intense cytoplasmic basophilia, which suggests a high content of RNA.

Epidermal and duct cells continue to form their respective cuticular products until day 11. Meanwhile zymogen cells enlarge greatly (Fig. 1d) and continue to synthesize DNA, as shown by incorporation of H^3-thymidine. The nuclei attain a high degree of ploidy (estimated as over 100 times the haploid level on the basis of nuclear size and staining density). Each nucleus contains as many as 100 large nucleoli, some of them 1 μm or more in diameter. The cytoplasm is intensely basophilic; in electron micrographs it displays an unusually high concentration of ribosomes, which are bound to membranes of the endoplasmic reticulum, and well-developed Golgi zones. Thus, the zymogen cells are poised to synthesize large amounts of protein for export.

Abruptly, on day 11 in *A. pernyi* or late day 9 in *A. polyphemus*, the zymogen cells begin the synthesis of cocoonase. As it is produced, the enzyme is sequestered in an apical vacuole (Fig. 1e), which appears homogeneous after fixation with glutaraldehyde or acrolein. Autoradiographic experiments with tritiated amino acids have shown that movement of labeled protein into the vacuole is complete within approximately 1.5 hours from the time of synthesis (*10*) so that the abrupt appearance of the vacuole reflects an abrupt shift in the pattern of protein synthesis.

Production of cocoonase is so rapid that the zymogen cells nearly double in height every 2 days, mostly because of expansion of the cocoonase-containing vacuole. On day 16 (Fig. 1f), the height of individual cells may reach 350 μm, of which more than 250 μm represents the vacuole. By comparison, the unspecialized epidermal cells measure only 5 to 10 μm in

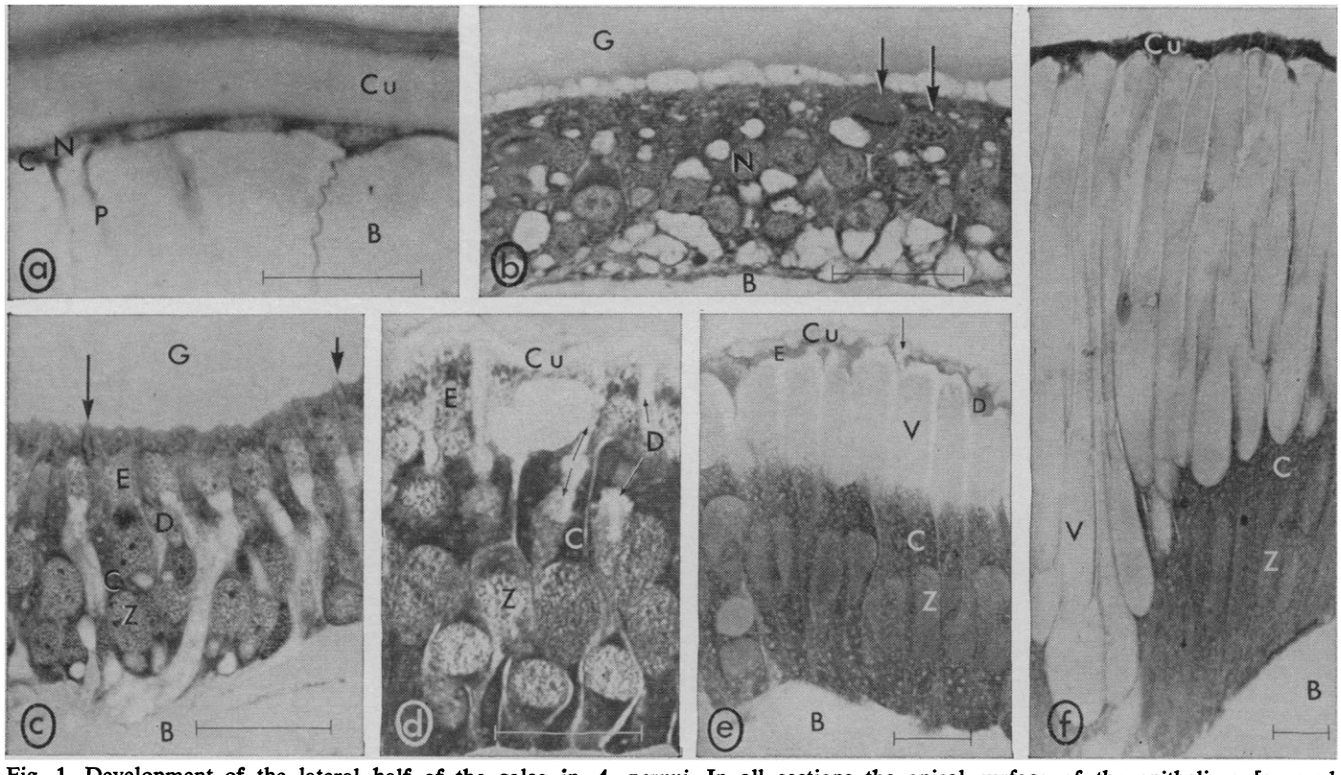

Fig. 1. Development of the lateral half of the galea in *A. pernyi*. In all sections the apical surface of the epithelium [covered by cuticle (*Cu*) or molting gel (*G*)] is uppermost; the blood (*B*) appears at the bottom of each picture. Sections 1 to 1.5 μm thick were stained with .05 percent toluidine blue (*5*); scale equals 20 μm. (a) Pupal gland, showing thick cuticle and squamous epithelial cells, each with flattened nucleus (*N*) and thin cytoplasm (*C*). (*P*) Cytoplasmic process directed toward the basement membrane (not in field). (b) Gland during the mitotic phase (day4). The squamous cells of Fig. 1a are growing and proliferating, giving a pseudostratified appearance to the epithelium. Arrows point to cells in prophase and metaphase. Molting gel separates the cuticle (not in field) from the detached epithelium. (c) Gland at day 7. Three distinct types of cells are identified by the letters *E, D,* and *Z* printed on their respective nuclei. The cuticle will be laid down shortly, just above the basophilic apical cytoplasm of the epidermal cells (*E*). Arrows point to developing cuticular ducts of the duct cells (*D*). Zymogen cells (*Z*) have intensely basophilic cytoplasm (*C*) and a large nucleus with several prominent nucleoli. (d) Gland late on day 10, just before cocoonase synthesis begins. Epidermal cells (*E*) are secreting the adult cuticle. Duct cells (*D*) are elaborating a system of cuticular tubules (arrows) opening to the surface and also projecting into zymogen cells (*Z*). The latter have markedly basophilic cytoplasm (*C*) and a large nucleus with many nucleoli—as many as 14 in a single section of a nucleus. (e) Gland early in the period of cocoonase synthesis (late day 12). The cocoonase vacuole (*V*) has appeared. Compare the size of *Z* cells with the *E* and *D* cells just below the cuticle. Arrow points to a duct. (f) Gland near the end of cocoonase synthesis (day 16).

diameter. On days 18 and 19 the vacuole gradually empties, as the enzyme solution is squeezed out onto the surface of the animal through the cuticular duct provided by a duct cell (*11*); shortly thereafter all the cells appear to degenerate. The cocoonase solution finally dries to a surprisingly pure, semicrystalline deposit (*1, 2*) on the surface of the animal. A few hours later, at the time of adult emergence, the enzyme redissolves in a copious buffer produced by labial glands (*12*) and softens the upper end of the cocoon, thus allowing the moth to escape (*1*).

In sum, the development of the lateral part of the galea can be divided into three phases, each lasting about 6 days. In the first phase (days 0 to 5), all cells fulfill the early functions of normal epidermis (retraction, secretion of molting gel) and then by mitosis and relative displacement diversify into three distinct cell types. In the second phase (days 5 to 11), two of these cell types are occupied with the synthesis of cuticular structures, while the developing zymogen cells increase their genetic material by endomitosis and accumulate the cytoplasmic machinery necessary for rapid protein synthesis. In the third phase (days 11 to 16), the zymogen cells synthesize large amounts of their characteristic protein cocoonase. With respect to this cell type, the first phase can be considered generative, the second differentiative, and the third differentiated. During the last phase, the zymogen cells account for almost all the cellular mass (and metabolism) of the galea.

In the zymogen cells DNA synthesis, as well as cytoplasmic growth, is maximum during the differentiative phase, as one might expect. Even in the fully differentiated state, however, replication of the genome continues: as late as day 11 in *A. polyphemus* (middle of day 12 in *A. pernyi*), most zymogen cells are still synthesizing DNA (Fig. 2b). By this time considerable cocoonase has already accumulated in the vacuole, and cocoonase synthesis accounts for more than half of the protein synthesis in each zymogen cell (Fig. 2a). The distinctive morphology of this tissue thus permits a direct demonstration that replication of DNA and synthesis of cell products characteristic of a differentiated state are not mutually exclusive processes (*13*).

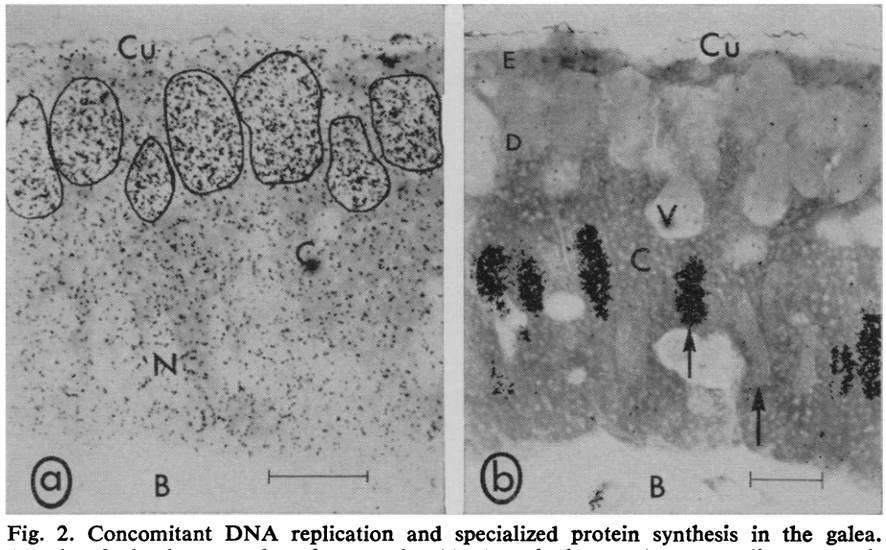

Fig. 2. Concomitant DNA replication and specialized protein synthesis in the galea. A pair of glands was taken from a day-11 *A. polyphemus* (corresponding approximately to a day-12 *A. pernyi*; compare with Fig. 1e). One gland (Fig. 2a) was incubated in vitro in Grace's medium containing 50 μc of H^3-leucine and 50 μc of H^3-lysine per milliliter (88 mc/mmole and 15 mc/mmole, respectively). Two hours later the gland was put in medium containing unlabeled amino acids; after 10 hours the gland was fixed and processed for autoradiography. The incubation period after labeling was sufficiently long (*10*) to allow complete transfer of the labeled cocoonase into the vacuole (outlined); cocoonase is the only protein present in the vacuole (*1*) and presumably accounts for all the radioactivity there. The distribution of grains indicates that 62 percent of the newly synthesized protein in these cells is cocoonase. The other galea (Fig. 2b) from the same animal was incubated for 10 hours in the presence of H^3-thymidine (50 μc/ml, 6 c/mmole) and was washed for 2 hours. Examination of twenty sections revealed that slightly more than half of the zymogen cell nuclei had synthesized DNA during this period. Arrows point to a labeled and an unlabeled nucleus. The autoradiogram in Fig. 2a was exposed for 5 days; that in Fig. 2b for 4 hours. Abbreviations as in Fig. 1.

The autoradiography was carried out on galeae maintained in Grace's medium (*14*) in a simple short-term organ culture (*10*); the conclusions were confirmed with studies in vivo. The availability of a convenient culture method greatly increases the usefulness of the galea for developmental studies, since it facilitates biochemical (*15*) as well as autoradiographic experiments. The zymogen cells have rapid metabolism and limited reserves, and consequently take up protein and nucleic acid precursors avidly (*10*).

The abrupt initiation of synthesis of a specific protein in the zymogen cells is of considerable interest. This process is a clear example of a developmental "switching" phenomenon and may lend itself to experimental analysis.

FOTIS C. KAFATOS

Biological Laboratories,
Harvard University,
Cambridge, Massachusetts 02138

NED FEDER

National Institutes of Health,
Bethesda, Maryland 20014

References and Notes

1. F. C. Kafatos and C. M. Williams, *Science* **146**, 538 (1964); F. C. Kafatos, thesis, Harvard University, Cambridge, Mass. (1965).
2. F. C. Kafatos, A. M. Tartakoff, J. H. Law, *J. Biol. Chem.* **242**, 1477 (1967); F. C. Kafatos, J. H. Law, A. M. Tartakoff, *ibid.*, p. 1488.
3. P. A. Marks and J. S. Kovach, *Curr. Top. Devel. Biol.* **1**, 213 (1966); F. H. Wilt, *Advance. Morphogenesis* **6**, 89 (1967); W. J. Rutter, N. K. Wessells, C. Grobstein, *Nat. Cancer Inst. Monogr.* **13**, 51 (1964); J. A. Stewart and J. Papaconstantinou, *J. Mol. Biol.* **29**, 357 (1967); R. Reeder and E. Bell, *J. Mol. Biol.* **23**, 577 (1967); V. B. Wigglesworth, *The Control of Growth and Form* (Cornell Univ. Press, Ithaca, N.Y., 1959).
4. C. M. Williams and P. L. Adkisson, *Biol. Bull.* **127**, 511 (1964).
5. N. Feder and T. P. O'Brien, *Amer. J. Bot.* **55**, 123 (1968).
6. R. DeG. Weevers, *J. Exp. Biol.* **44**, 163 (1966).
7. Like all appendages in the pupa, the galeae are reflected back against the body wall and are cemented in place, lodged among the antennae, legs, and labial palps. Only their ventral surfaces are exposed, appearing as two triangles 5 to 6 mm long and 2 mm wide.
8. J. V. Passonneau and C. M. Williams, *J. Exp. Biol.* **30**, 545 (1953).
9. While in the vacuole, cocoonase is apparently a zymogen which is activated only as it is conveyed to the surface; fresh galeal homogenates are inactive in proteolytic assays even on day 16 but become active upon prolonged incubation at room temperature.
10. F. C. Kafatos and J. Reich, *Proc. Nat. Acad. Sci. U.S.*, in press.
11. On day 17 (day 15 in *A. polyphemus*) an air space appears around the galea, replacing the molting fluid, which is resorbed after digesting the pupal endocuticle. The cocoonase solution is expelled into this space.
12. F. C. Kafatos, *J. Exp. Biol.* **48**, 435 (1968).
13. F. E. Stockdale and H. Holtzer, *Exp. Cell Res.* **24**, 508 (1961).
14. T. D. C. Grace, *Nature* **195**, 788 (1962).
15. F. C. Kafatos, *Proc. Nat. Acad. Sci. U.S.* **59**, 1251 (1968).

16. Supported in part by NSF grant GB-4171 to F.C.K., PHS grant GM-08139 to N.F., and NSF grant GB-3232 to C. M. Williams. We thank Prof. C. M. Williams for assistance and encouragement, and Susan Hall Branson and E. B. Boatner for technical assistance.

20 June 1968